THE METABOLISM OF SAPROPHYTIC HUMAN TUBERCLE BACILLI IN PLAIN, DEXTROSE, MANNITE AND GLYCERIN BROTHS

STUDIES IN ACID-FAST BACTERIA. I

A. I. KENDALL, A. A. DAY, AND A. W. WALKER

Reprinted from
The Journal of Infectious Diseases, Vol. 15, No. 3, Nov. 1914, pp. 417-422

THE METABOLISM OF SAPROPHYTIC HUMAN TUBERCLE BACILLI IN PLAIN, DEXTROSE, MANNITE, AND GLYCERIN BROTHS

STUDIES IN ACID-FAST BACTERIA. I

A. I. Kendall, A. A. Day, and A. W. Walker

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

One of the noteworthy contributions to the chemistry of the tubercle bacillus was the discovery by Theobald Smith[1] that human tubercle bacilli produce a terminal acid reaction in nutrient glycerin broth, contrasting in this respect with bovine tubercle bacilli, cultures of which tend to an alkaline reaction in the same media. Attention was directed by him to slight variations in this reaction of the human bacilli; if the medium is not favorable for the luxuriant growth of these organisms, this terminal acidity may be lessened or even be absent. Furthermore, as he pointed out, this acid reaction is developed only when the glycerin content of the broth is 1 percent or more; a concentration of at least 2 percent of glycerin is necessary for the maximum acidity. Again, this acidity is a differential acidity, for alkaline products appear to be formed in glycerin broth coincidently with the acid products derived from the glycerin. A differential excess of acid, rather than a purely fermentative decomposition of glycerin, is responsible for this noteworthy difference between the human and bovine types of tubercle bacillus. The latter organism does not appear to form acid from glycerin. The development of alkalinity, probably derived from protein constituents of the broth, is brought out sharply when bovine and human bacilli, respectively, are grown in broth cultures containing no glycerin. In this medium, altho the growth is less luxuriant than in glycerin broth, the reaction becomes progressively alkaline in both instances.

This work is important from another point of view. Prior to the publication of these studies, investigators, almost without exception, assumed that tubercle bacilli could not be cultivated in artificial media unless glycerin was present. Some observers have gone so far as to

1. Trans. Assn. Am. Phys., 1903, 18, p. 108; Jour Med. Research, 1905, 13, p. 253; Am. Jour. Med. Sci., 1904, 128, p. 216.

state dogmatically that glycerin was a *sine qua non* for the artificial cultivation of these organisms. The observations mentioned above show definitely that the majority of strains of tubercle bacilli will develop, altho much less luxuriantly, even in plain nutrient broth. These organisms, therefore, are not obligately glycerophilic.

Dextrose, or muscle sugar, appears to play no part whatsoever in the development of tubercle bacilli in glycerin broth, for the amount of dextrose remains the same in broth after prolonged cultivation of these organisms in it as in uninoculated broth, as shown by the fermentation test with the colon bacillus. These observations were soon confirmed, and they were apparently accepted without question by Koch as the only practical cultural differentiation between human and bovine bacilli.

From time to time strains of tubercle bacilli have been isolated from man which appear to be intermediate between the human and bovine types when judged by this criterion, but for the most part subsequent investigation has shown that these few intermediate strains revert, after prolonged cultivation, to one type or the other, or they have been definitely referred to one of the other types by careful animal experiments. A few strains, however, appear to be exceptional in this respect.

The experiments here recorded were undertaken with the specific purpose of studying the gross metabolism of tubercle bacilli in artificial media, particularly with reference to the influence of glycerin on the metabolism of these organisms.

The organisms studied in this connection were two strains of rapidly growing human tubercle bacilli; one of these, "W", was kindly sent us by Professor Wherry of the University of Cincinnati; the other, "597", from the Museum of Natural History, New York, by Professor Winslow. Culture "W" is a descendant of a culture from Koch's laboratory, brought to this country by Professor Vaughan of the University of Michigan. It is avirulent for guinea-pigs. Culture "597" will produce tuberculosis in guinea-pigs if relatively large doses are injected intraperitoneally. It should be emphasized that these cultures, particularly "W", in virtue of their avirulence for guinea-pigs, might be questioned with respect to their identity as human tubercle bacilli.

Further observations along similar lines with human bacilli of known virulence, however, which will be presented later, have given reactions qualitatively identical with those mentioned above, and the study of a considerable series of acid-fast bacilli, not tubercle bacilli, have shown unmistakable cultural differences which appear to separate them from these avirulent tubercle bacilli.

Rapidly growing tubercle bacilli offer advantages for chemical study which are obvious; their relatively active development leads to an early accumulation of decomposition products in measurable amounts, and these products are less influenced by parallel recessive changes due to autolysis of the bacilli themselves than appears to be the case with the highly virulent, slowly growing strains.

The media selected for this study comprise nutrient sugar-free broth made from meat juice as a basis. Portions of this broth were re-enforced by 1 percent dextrose, 1 percent mannite, and 3 percent of glycerin, respectively, as additional sources of carbon. As in previous experiments,[2] several flasks of the same size and shape [250 c.c. Erlenmeyer flasks], each containing 100 c.c. of the medium, were prepared at the same time and inoculated and incubated under similar conditions of temperature and moisture. The methods of analysis used have also been described in detail elsewhere[3] and need not be referred to here. The determinations comprise: changes in reaction, using alizarin, neutral red, and phenolphthalein as indicators; and the production of ammonia and a parallel study of the morphology, using the Ziehl-Neelsen stain. The stains for a given series were all made at the same time and subjected to the same degree of heat, decolorization, etc. to obviate errors in this direction as far as possible. The results based on the initial volume of 100 c.c. of media follow, the reaction being expressed in terms of cubic centimeters of normal acid or alkali per hundred cubic centimeters of broth, the ammonia as milligrams of nitrogen per 10 c.c. of broth. Several parallel experiments, some in duplicate, were made at different times in different lots of media. These are included to indicate the approximate limits of variation which may be expected.

The only noteworthy features in the morphology and staining reactions were a change about the end of the fourth week from the typical, rather long, slender rod to a short, thick rod, and a moderate proportion of non-acid-fast rods, typical, however, morphologically during the first two weeks of growth. These short, thick rods resemble strikingly the bovine bacillus. The experiments, however, do not suggest that a further cultivation of these short rods would result in a permanent change in morphology from organisms resembling the human type of the bovine bacillus to that of the bovine. The change from partial acid-fastness to complete acid-fastness seems to be somewhat closely associated with a recession in chemical activity. Wolbach and Ernst[4] have shown that young cultures of tubercle bacilli exhibit a certain degree of non-acid-fastness. Neither dextrose, mannite, nor glycerin spare protein to any such degree as dextrose, for example, spares protein under similar conditions with a great majority of the commonly met with bacteria.

2. Kendall, Day and Walker, Jour. Am. Chem. Soc., 1912, 35, p. 14.
3. Kendall and Farmer, Jour. Biol. Chem., 1912, 12, p. 13.
4. Jour. Med. Research, 1903, 10, p. 313.

Throughout these experiments the growth in plain broth was distinctly less luxuriant and less extensive than that occurring in dextrose, mannite, or glycerin media. The growths in the later media were of about equal magnitude. After a period of alkalinity the reaction gradually becomes acid in glycerin broth, and progressively alkaline in plain, dextrose, and mannite broths. This is in accord with Theobald Smith's observations mentioned above. The changes in ammonia production would indicate that this development of acid is not to be interpreted as

TABLE 1

Metabolism of Tubercle Bacilli in Broth

Bacillus tuber-culosis	Days	Plain Broth					Dextrose Broth				
		Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent
	4	—1.10	0.00	—0.20	5.6	2.50	0.00	0.00	—0.20	4.9	2.50
	6	—2.50	—0.50	—0.50	10.5	4.69	—0.20	0.00	—0.10	5.6	2.85
597	13	—3.40	—1.20	—0.90	26.6	11.87	—1.80	—0.50	—0.10	23.1	11.80
	20	—3.00	—1.40	—1.10	21.0	9.37	—3.20	—1.50	—0.80	30.8	15.70
	27	—3.30	—1.50	—2.00	15.4	6.88	—2.60	—1.70	—1.00	21.7	11.60
	34	—2.10	—1.50	—0.90	16.1	7.23	—1.70	—1.20	—0.90	15.4	7.85
	4	—1.10	0.00	—0.30	2.8	1.25	—0.20	0.00	—0.20	4.9	2.50
	6	—2.30	—0.40	—0.50	8.4	3.75	—0.20	0.00	—0.20	4.9	2.50
W	13	—2.80	—1.10	—0.80	25.2	11.25	—2.70	—1.20	—0.50	23.1	11.80
	20	—3.20	—1.50	—0.90	26.6	11.88	—2.80	—1.50	—0.80	23.1	11.80
	27	—2.90	—1.40	—0.60	23.8	10.63	—2.10	—1.30	—0.80	24.5	12.50
	34	—1.10	—1.50	—0.90	16.8	7.50	—1.40	—1.20	—0.60	11.2	5.72
	1	—0.30	0.00	—0.20	2.1	0.68	—0.30	—0.10	0.00	2.1	0.68
	4	—1.60	—0.90	—0.40	5.6	1.81	—1.50	—0.60	—0.20	1.4	0.45
W1	7	—3.30	—1.40	—1.50	22.4	7.30	—3.40	—1.40	—0.70	23.1	7.50
	14	—3.60	—2.00	—1.40	26.6	8.62	—3.70	—2.20	—1.10	24.5	7.98
	21	—3.60	—2.30	—1.30	24.5	7.98	—3.00	—2.20	—0.90	23.8	7.72
	28	—3.20	—2.20	—1.30	14.7	4.77	—3.00	—2.40	—1.40	23.8	7.72
	35	—2.90	—1.40	—1.30	8.4	2.79	—2.60	—1.60	—1.60	7.0	2.33
	1	0.00	+0.10	0.00	0.70	0.24	—0.20	—0.20	—0.10	0.70	0.23
	4	—1.10	—0.50	—0.30	4.2	1.46	—1.60	—0.90	—0.50	5.6	1.86
W2	7	—2.70	—1.40	—1.10	17.5	6.10	—3.00	—1.40	—0.80	21.0	6.98
	14	—2.90	—2.00	—1.20	18.2	6.35	—3.60	—2.60	—1.10	30.8	10.70
	21	—2.80	—1.60	—1.20	14.7	5.13	—3.10	—2.00	—1.60	14.0	4.65
	28	—2.60	—1.50	—1.30	9.1	3.17	—2.60	—2.20	—1.70	9.1	3.02

a sparing action of the glycerin for protein. Perhaps the most striking feature of the metabolism is the rather general recession of the ammonia. Ammonia production reaches its maximum about the second or third week of incubation, and then gradually diminishes, so that the final amount of ammonia detectable in the cultures is usually much less than that observed at the height of vegetative activity. This phenomenon has not been observed in cultures of other bacteria which have been studied similarly. The reason for this recession is not apparent. Several possible explanations present themselves. Loss of ammonia

by volatilization is not very probable, for the solution is not unduly alkaline and the small amount of ammonia formed, taken into consideration with the great solubility of this substance in water, would seem to eliminate this possibility. With our present-day knowledge of autolysis of bacterial cells it would appear that the ammonia should increase rather than diminish if this were the chief cause. In this connection it should be stated that the pellicles showed no visible diminution in size, but this does not exclude the possibility that the contents

TABLE 1—(*Continued*)

Metabolism of Tubercle Bacilli in Broth

Bacillus tuber-culosis	Days	Mannite Broth					Glycerin Broth				
		Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent
	4	—0.40	+0.10	—0.10	—2.1	—0.97	+0.50	+0.30	+0.20	0.00	0.00
		—0.30	+0.10	—0.10	—2.1	—0.97	—0.50	+0.10	+0.10	6.3	3.10
597	13	—1.90	—0.60	+0.20	21.7	10.00	—1.00	—0.10	+0.40	24.5	12.05
	20	—2.10	—0.90	—0.40	35.2	14.85	—1.30	—0.30	+0.60	35.0	17.25
	27	—2.60	—1.20	—0.60	29.4	15.55	—1.00	—1.10	+0.40	39.9	19.65
	34	—2.00	—1.40	—0.20	29.4	15.55	—0.80	—0.10	+1.20	44.1	21.70
	4	—0.50	+0.20	—0.10	0.00	0.00	—0.80	+0.10	—0.30	2.1	1.03
	6	—0.70	+0.10	0.00	4.2	1.93	—0.30	+0.20	0.00	2.1	1.03
W	13	—3.00	—1.30	—0.80	34.3	15.80	0.00	+0.30	+0.50	19.6	9.68
	20	—2.70	—1.50	—1.00	25.9	11.92	—0.50	+0.20	+0.80	27.3	13.45
	27	—2.30	—1.50	—0.90	21.7	10.00	—1.90	—0.10	+0.40	35.7	17.60
	34	—1.60	—1.20	—1.00	13.3	6.13	—0.90	—0.30	+0.40	35.7	17.60
	1	—0.30	—0.30	—0.20	1.4	0.47	—0.20	0.00	—0.30	1.4	0.47
	4	—1.30	—0.40	—0.50	2.1	0.70	—1.50	—0.60	—0.30	5.6	1.86
W1	7	—2.40	—0.80	—0.40	17.5	5.81	—2.20	—0.80	—0.30	16.8	5.58
	14	—3.70	—2.10	—1.20	35.7	11.87	—2.30	—1.20	—0.50	23.8	7.90
	21	—3.30	—3.00	—0.90	34.3	11.40	—2.30	—1.60	+0.10	26.6	8.85
	28	—3.10	—3.20	—1.60	26.6	8.82	—2.70	—1.70	+0.40	27.3	9.08
	35	—3.30	—1.50	—0.90	28.7	9.51	—2.30	—1.30	+0.70	7.7	2.55
	1	—0.30	—0.20	0.00	0.00	0.00	—0.10	—0.10	+0.10	—0.70	—0.24
	4	—2.90	—1.40	0.00	7.7	2.62	—1.20	—0.60	—0.10	9.1	3.17
W2	7	—3.40	—2.20	—1.10	23.8	8.1	—1.30	—1.10	—0.10	18.2	6.34
	14	—4.20	—2.70	—1.60	17.5	5.95	—2.40	—1.90	+0.70	32.2	11.2
	21	—2.70	—2.00	—1.50	14.0	4.76	—1.40	—1.20	+0.10	21.0	7.3
	28	—2.10	—2.10	—1.60	13.3	4.53	—1.80	—1.30	+0.20	21.7	7.53

of the bacteria may have become soluble, leaving their skeletons, as it were, intact.

The fundamental composition of media made from meat juice and peptone must be extremely complex, and it is conceivable that certain substances, perhaps of the nature of fats, lipoids, or their derivatives, might play a part. These substances are present undoubtedly in small amounts in ordinary media. Tubercle bacilli contain considerable amounts of fats and their derivatives in their bodies, and it is conceivable that these substances play some part in this reaction.

CONCLUSIONS

Young, rapidly-growing tubercle bacilli appear to be, in part at least, non-acid-fast.

The strain of avirulent tubercle bacilli studied here exhibit the Theobald Smith reaction characteristic of the growth of human tubercle bacilli in glycerin broth.

Neither dextrose, mannite, nor glycerin appears to exert any marked sparing action for the protein constituents of ordinary media.

Ammonia accumulates rather rapidly during the first, second, and third weeks of growth of tubercle bacilli in plain, dextrose, mannite, and glycerin broths, followed by a definite well-marked recession, during which this ammonia detectable in the media gradually diminishes in amount. The cause of this recession is unknown.

THE METABOLISM OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

STUDIES IN ACID-FAST BACTERIA. II

A. I. KENDALL,
A. A. DAY,
AND
A. W. WALKER

Reprinted from
THE JOURNAL OF INFECTIOUS DISEASES, Vol. 15, No. 3, Nov. 1914, pp. 423-427

THE METABOLISM OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

STUDIES IN ACID-FAST BACTERIA. II

A. I. Kendall, A. A. Day, and A. W. Walker

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

The study of the metabolism of two rapidly growing, avirulent tubercle bacilli of human origin, in plain, dextrose, mannite, and glycerin nutrient broths, showed a consistent and well-defined progressive breakdown of the protein constituents in all of these media which reached its maximum between the second and the third week (see preceding article). The breakdown of protein was measured by the increase of ammonia. About the third week, ammonia production reached its maximum and then the amount of ammonia, detectable in the media by the method used, showed a definite, progressive recession, so that at the end of five or six weeks the amount of ammonia, altho greater than that in uninoculated controls, was decidedly less than the maximum amount which was found after about three weeks' incubation. No definite explanation for this recession was apparent.

Nutrient broth, as it is usually prepared, contains small amounts of fats, fat derivatives, and lipoids, and it is conceivable that some of these lipoidal substances may play a part in at least the initial phase of this reaction. Lipoids could hardly explain all of the phenomena involved in the ammonia curve, however. Some of these lipoidal substances have attracted considerable attention, particularly in connection with the growth of the tubercle bacillus *in vitro* and in the body. Von Eisler and Laub[1] studied the lipoidal content of the serum of ninety-five tuberculous patients and found it low in all. There was no relation between the decrease of the lipoidal content of the blood and the temperature curve. They found that the cholesterin esters, but not cholesterin itself, were decreased in amount. Deycke and Much[2] and Sieber

1. Wien. klin. Wchnschr., 1913, 26, p. 968.
2. München. med. Wchnschr., 1909, 56, p. 1986; Berl. klin. Wchnschr., 1910, 47, p. 1933; Centralbl. f. Bakteriol., Abt. I, Orig. I, 1910, 54, p. 342.

and Metalnikoff[3] claim that lecithin, neurin, and cholin dissolve tubercle bacilli in the test-tube, while Löwenstein and Beyer[4] deny that the lecithin has any bactericidal action on the tubercle bacillus. These latter observers believe that the liberation of acid, incidental to the decomposition of lecithin, is the cause of the destruction of the organisms.

In order to determine what part, if any, these lipoidal substances, in the amounts in which they occur in ordinary media, might play in the metabolism of tubercle bacilli, media were made up from ingredients in which these substances were definitely and quantitatively eliminated. This was accomplished as follows:

Fifteen grams of Fairchild's peptone were extracted for two weeks with ether, two weeks with alcohol, two weeks with acetone, and ten days with petroleum ether, respectively — in a Soxhlet extractor, the successive extrac-

TABLE 1

Metabolism of Tubercle Bacilli in Broth Free from Lipoids and Fatty Substances

Bacillus tuberculosis	Days	Plain Broth A					Plain Broth B				
		Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent	Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent
597	3	—0.60	—1.00	—0.90	4.2	3.15	—1.40	—1.30	—1.40	11.9	5.32
	7	—1.70	—1.80	—1.30	29.4	22.1	—3.30	—2.80	—1.90	52.5	23.5
	14	—1.90	—1.90	—1.10	38.5	29.0	—3.00	—2.70	—1.70	65.1	29.0
	21	—2.10	—2.10	—1.40	35.0	26.3	—3.80	—3.50	—2.00	60.9	27.2
	28	—1.80	—2.10	—1.10	28.7	21.6	—3.80	—2.90	—1.70	48.3	21.5
W	3	—0.30	—0.50	—0.50	1.4	1.45	—1.40	—1.00	—1.30	11.9	5.3
	7	—1.80	—1.70	—1.30	23.8	17.90	—3.60	—2.90	—1.70	49.7	22.2
	14	—1.60	—1.80	—1.20	37.8	28.4	—2.80	—2.80	—1.80	64.4	28.7
	21	—2.00	—2.20	—1.30	35.0	26.3	—3.70	—3.50	—1.90	60.2	26.9
	28	—1.70	—1.90	—1.30	28.7	21.6	—3.50	—2.80	—1.90	52.5	23.4

tions occurring at intervals of about 12 minutes; these extractions were continued for about six hours daily and for six days per week. Considering the large amount of solvent which bathed this peptone, it is fair to assume that these substances were removed quantitatively. This peptone, which had been extracted, was made into broth by the addition of distilled water; one portion consisting of peptone alone; one portion containing peptone and 1 percent of dextrose; and a third portion containing 3 percent of glycerin in addition to the peptone. Three grams of Na_2HPO_4 and five grams of NaCl per liter were added to each kind of medium. A fourth lot of broth, containing unextracted peptone and the same salts, but with no additional source of carbon, was prepared under the same conditions to serve as a control.

All of the utensils used in the preparation of these fat-free media were cleaned first with alkaline potassium permanganate, then with oxalic acid,

3. Centralbl. f. Bakteriol., Abt. I, Orig., 1910, 54, p. 349.
4. Ibid., 56, p. 160.

then with chromic acid, and washed thoroughly in water and then with distilled water, so that it is certain that no foreign substance could have been introduced into the media during the process of manufacture.

The media thus prepared contained no meat extract or meat juice. It is impossible to rule out the presence of minute traces of dextrose or muscle sugar. These media were distributed in flasks in the usual manner, 100 c.c. per flask, and autoclaved at the same time and inoculated respectively with the two strains of rapidly growing avirulent tubercle bacilli, W. and 597.

The determinations were made in precisely the same manner as those in Study I. The media are designated for purposes of convenience Plain Broth A, Plain Broth B, Dextrose Broth A, and Glycerin Broth A. Plain Broth B, it will be remembered, was made from peptone which had not been extracted. Plain Broth A, Dextrose, and Glycerin Broths were prepared from the peptone which had been extracted as outlined.

Several features in the metabolism of these organisms are noteworthy (Table 1). Plain Broth A (uninoculated) contained 133 mg. of nitrogen per 100 c.c. of broth, while Broth B, which had not been

TABLE 1—(*Continued*)

METABOLISM OF TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

Bacillus tuberculosis	Days	Dextrose Broth A					Glycerin Broth A				
		Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Alizarin	Neutral Red	Phenolphthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent
	3	—0.70	—0.70	—1.30	6.3	4.50	—0.50	—0.70	—0.60	2.10	1.58
	7	—.040	—0.40	—1.20	6.3	4.50	0.00	—0.30	—0.60	3.5	2.63
597	14	—1.00	—1.00	—1.50	29.4	21.0	0.20	—0.50	—0.80	—0.70	—0.52
	21	—1.50	—1.60	—1.50	23.1	16.5	0.20	—0.60	—0.70	—2.10	—1.58
	28	—1.40	—1.50	—1.40	23.8	17.0	0.20	—0.60	—0.90	—2.80	—2.10
	3	—0.60	—0.70	—1.20	5.6	4.0	—0.30	—0.40	—0.50	3.5	2.63
	7	—0.60	—0.60	—1.40	1.4	1.0	—0.10	—0.40	—0.80	1.4	1.05
W	14	—0.90	—1.50	—1.40	17.5	12.5	0.10	—0.70	—0.80	—2.8	—2.1
	21	—1.50	—1.70	—1.50	23.1	16.5	0.10	—0.60	—0.80	—2.8	—2.1
	28	—1.50	—1.40	—1.40	23.8	17.0	0.10	—0.80	—0.90	—2.8	—2.1

extracted, contained 224 mg. of nitrogen in the same volume. That is to say, during the process of extraction a considerable amount of nitrogenous substance had been removed coincidently with the removal of the fatty substances. The nature of these nitrogenous substances is unknown. In Plain Broth A and Plain Broth B the ammonia production reaches its maximum at the end of the fourteenth day, at which time it is about 29 percent of the total nitrogen of the media. Notwithstanding the fact that the percentage of ammonia to total nitrogen is the same in both media, the actual amount of ammonia in Plain Broth B, that is, the unextracted broth, is much greater, being, roughly, in the proportion of 65 mg. to 38 mg. It would appear that the

removal of certain nitrogenous substances from Plain Broth A did not materially influence the percentage of protein breakdown as compared with Broth B, and the results suggest, furthermore, that the additional nitrogenous content of Broth B was broken down readily. Plain Broth B was distinctly more alkaline in reaction than Plain Broth A, and this increased alkalinity can be explained tentatively on the basis of the greater production of ammonia in this medium. In the dextrose broth, the amount of ammonia produced was distinctly less than that in the corresponding plain broth. This might be interpreted as a sparing action of the dextrose for the protein constituents of the broth, but this sparing action is far less marked, if indeed it be a sparing action, than is the case with ordinary bacteria studied under the same conditions.[5] In glycerin broth, after a slight initial increase in ammonia amounting to about 2 percent of the total nitrogen of the medium, the ammonia appears to decrease in amount, so that at the end of the experiments it is less than that contained in the uninoculated media. At the end of the second week, the glycerin broth cultures of both strains of the tubercle bacillus were found to be slightly viscid, and by the end of the fourth week, this viscosity was very marked. The reaction of the medium to phenolphthalein becomes progressively alkaline in spite of this decrease in ammonia. There is no satisfactory explanation for this phenomenon available at the present time. It is conceivable that at least some of this ammonia is tied up in the bodies of the bacteria, and inasmuch as the organisms studied in this connection form firm tenacious pellicles, leaving the medium beneath them perfectly clear and free from bacteria, it was a comparatively simple matter to make a determination of the total nitrogen of the clear underlying broth. The analyses follow.

Organism	Days	Plain Broth A		Plain Broth B		Dextrose Broth A		Glycerin Broth A	
		Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss
Control	—	133	000	224	000	140	000	133	000
W	28	98	26.3	147	34.3	98	30.0	56	57.9
597	28	77	42.0	154	31.3	96	35.0	42	68.4

5. Kendall, Day and Walker, Jour. Am. Chem. Soc., 1913, 35, p. 1208.

The results show that a very considerable proportion of the total nitrogen in the medium is, apparently, tied up in the bodies of the bacteria. It might be objected at this point that some of this nitrogen may have escaped from the medium, as ammonia, by evaporation, escape perhaps being facilitated by the pellicle floating on the surface. Ammonia, in the amounts produced in these media, almost certainly could not evaporate from the free surface of the medium because of the great affinity of ammonia for water. It is possible, however, that the presence of a pellicle might result in a direct "exhaling" of ammonia into the air, the pellicle acting as a barrier to its reabsorption. This pellicle, it should be remembered, is somewhat dry, and while some loss may have taken place, the amount is probably insignificant when compared with the total amount of nitrogen of the medium. It will be observed that in glycerin-broth there was a much greater amount of nitrogen in the pellicle than is the case with the other media. This might be accounted for on the basis of a difference in the luxuriance of the growth, and it is a fact that the pellicles formed, respectively, on Plain Broths A and B are thinner and less extensive than the one formed in glycerin. The pellicle formed in dextrose, however, appears to be quite as dense as that formed in glycerin. It is a noteworthy fact that even the organisms grown in the Plain Broth A, which, theoretically at least, is free from all fats, fatty derivatives, and lipoids, are acid-fast.

While these experiments do not by any means prove that the substance or substances conferring acid-fastness on these organisms are derived from protein derivatives alone, yet it would seem that an experiment of this sort carried out under similar conditions, with especial emphasis on the fat and wax content of the organisms, would throw some definite light on the physiology of the formation of fats and waxes from protein.

The experiments do not explain the recession of ammonia which was noticed in broths containing small amounts of fats and lipoids, and it is probable that these substances do not play any material part in this recession. It is worthy of note that the reaction curve of these organisms in glycerin broth does not conform to the Theobald Smith curve for human tubercle bacilli, the reaction produced being progressively alkaline instead of becoming acid. The composition of the glycerin broth in which these observations were made, however, is so different from that usually employed for this purpose that the results are not at all comparable in the two instances.

THE METABOLISM OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN A MODIFIED USCHINSKY MEDIUM

STUDIES IN ACID-FAST BACTERIA. III

A. I. KENDALL,
A. A. DAY,
AND
A. W. WALKER

Reprinted from
THE JOURNAL OF INFECTIOUS DISEASES, Vol. 15, No. 3, Nov. 1914, pp. 428-432

THE METABOLISM OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN A MODIFIED USCHINSKY MEDIUM

STUDIES IN ACID-FAST BACTERIA. III

A. I. KENDALL, A. A. DAY AND A. W. WALKER

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

In the previous articles the recession of ammonia was observed in old cultures of avirulent, rapidly growing cultures of tubercle bacilli. The cause of this recession was unknown, but probably it is not attributable to small amounts of lipoids or fats.

The present study was made to determine the incidence of this reaction in media of very simple composition. The experiments of Proskauer and Beck,[1] which have since been repeated by many observers,[2] have shown that the human tubercle bacillus can grow in media of relatively simple composition. These media would seem to offer advantages for the study of the recession of ammonia which we have observed. This advantage lies in the fact that it is possible to provide, as a source of nitrogen, a chemical compound the composition of which is definitely known. For the purpose of this investigation, a medium was made consisting of 4 gm. of asparagin, 2 gm. of di-sodium hydrogen phosphate, and 5 gm. of NaCl to the liter of distilled water, as a basis. This medium was divided into three parts: to one of which was added 1 percent of dextrose; to a second, 1 percent of mannite, and to a third, 3 percent of glycerin, as additional sources of carbon. They were then sterilized under parallel conditions, in 100 c.c. amounts. All of the utensils, which were used in the preparation of these media, were thoroughly freed from all organic matter. It will be seen that the source of nitrogen in this medium is asparagin. The source of carbon is selective, the organisms having a choice of the carbon from the asparagin or from dextrose, mannite, or glycerin, respectively. An attempt was made to grow the organisms in the asparagin solution without any additional source of carbon, but this was unsuccessful.

1. Ztschr. f. Hyg. u. Infectionskrankh., 1894, 18, p. 128.
2. Frankel, Hyg. Rundschau, 1894, p. 769; Frouin, Compt. rend. Soc. de biol., 1910, 68, p. 915; Lowenstein, Centralbl. f. Bakteriol., Abt. I, Orig., 1913, 68, p. 591; Wherry, Ibid., 70, p. 115; and Jour. Infect. Dis., 1913, 13, p. 144.

TABLE 1

METABOLISM OF TUBERCLE BACILLI IN A MODIFIED USCHINSKY MEDIUM

Bacillus tuberculosis	Days	Dextrose Broth					Mannite Broth					Glycerin Broth				
		Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mgs. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent
WI	7	0.00	+0.20	—0.10	4.9	6.60	—0.30	+0.20	0.00	11.2	12.5	—0.20	+0.10	+0.20	4.2	6.52
	14	—0.10	+0.20	+0.20	4.9	6.60	—0.30	+0.10	+0.20	25.9	39.4	0.00	+0.10	+0.10	4.9	7.61
	21	—0.20	+0.10	+0.40	23.8	32.1	—0.30	—0.10	+0.20	30.8	46.8	—0.10	—0.10	+0.30	9.8	15.2
	28	—0.20	0.00	+0.30	36.4	49.1	—0.50	—0.20	+0.20	39.9	60.3	—0.20	—0.20	+0.30	28.0	43.4
	35	—0.20	+0.40	+0.20	30.8	41.5	—0.30	+0.10	+0.30	30.8	46.8	—0.20	0.00	0.00	30.8	47.8
	42	—0.10	+0.10	+0.10	34.3	46.3	—0.60	—0.10	+0.10	31.5	48.0	—0.20	—0.10	+0.10	33.6	52.1
WII	7	0.00	+0.20	+0.10	4.2	4.55	—0.10	+0.20	+0.10	7.7	8.6	—0.10	+0.20	+0.30	4.2	6.52
	14	—0.10	+0.10	+0.10	6.3	8.49	—0.30	0.00	+0.30	23.1	41.8	—0.10	0.00	+0.20	6.3	9.78
	21	—0.20	0.00	0.00	14.0	18.9	—0.30	—0.20	+0.20	31.5	48.0	—0.10	—0.10	+0.20	14.0	21.8
	28	—0.20	+0.10	+0.20	36.4	49.1	—0.60	—0.20	+0.10	37.8	57.5	—0.20	—0.10	+0.20	27.3	42.4
	35	—0.20	+0.20	+0.30	31.5	42.4	—0.40	+0.10	0.00	30.8	46.8	—0.20	+0.10	+0.10	32.2	50.0
	42	—0.20	+0.10	+0.30	33.6	45.3	—0.40	0.00	+0.10	33.6	51.1	—0.20	0.00	+0.10	35.0	54.4
597I	7	0.00	+0.20	+0.10	3.5	4.87	—0.20	+0.20	+0.10	7.0	7.81	—0.10	+0.10	0.00	2.8	4.35
	14	0.00	+0.30	—0.10	4.9	6.60	—0.40	+0.10	0.00	21.0	31.9	0.00	+0.10	+0.20	2.1	3.26
	21	—0.10	0.00	+0.50	18.2	24.5	—0.50	—0.20	+0.10	30.1	45.8	—0.10	0.00	+0.20	23.8	37.6
	28	—0.10	+0.10	+0.50	32.9	44.3	—0.30	—0.10	+0.10	39.9	60.3	—0.20	—0.10	+0.20	25.2	39.3
	35	0.00	+0.30	+0.50	30.1	40.5	—0.30	+0.10	+0.10	32.2	49.0	—0.10	0.00	+0.20	28.0	43.4
	42	—0.10	0.00	+0.20	32.2	43.4	—0.30	—0.10	0.00	31.5	48.0	—0.10	0.00	+0.40	31.5	44.7
597II	7	0.00	+0.10	+0.30	4.2	4.55	—0.20	+0.20	+0.20	7.0	7.81	0.00	0.00	+0.10	2.1	3.26
	14	0.00	+0.30	0.00	5.6	7.54	—0.40	—0.10	+0.10	21.0	31.9	0.00	0.00	+0.30	2.1	3.26
	21	—0.10	0.00	+0.30	17.5	23.6	—0.30	—0.20	+0.10	30.1	45.8	—0.05	0.00	+0.30	10.5	16.3
	28	—0.10	+0.10	+0.30	31.5	42.4	—0.40	—0.20	+0.10	39.9	60.3	—0.05	—0.20	+0.30	25.2	39.3
	35	—0.20	+0.20	+0.30	31.5	42.4	—0.10	+0.20	+0.10	31.5	48.0	—0.05	0.00	+0.10	23.8	37.6
	42	—0.10	0.00	+0.30	31.5	42.4	—0.30	—0.10	+0.20	31.5	48.0	—0.10	—0.10	+0.30	32.2	50.0

The determinations were made in duplicate at weekly intervals for six weeks, and, with the exception of glycerin, the same recession of ammonia appears, as was noted in the previous experiments. The glycerin does not show this recession. The extent of the recession is somewhat less marked than in peptone-containing media, but the growth, it should be noted parenthetically, was much less luxuriant. Nevertheless, the changes induced in these media, both in reaction and in the decomposition of the nitrogenous constituents, were of sufficient magnitude to warrant the conclusion that the bacteria grew with moderate luxuriance. It was observed previously (Study II) in a medium containing peptone, which had been thoroughly extracted with organic solvents, that the reaction became progressively alkaline in glycerin. In the asparagin medium containing glycerin, the reaction was slightly, but consistently, acid to phenolphthalein. This was also the case in the dextrose and mannite media. The reaction with alizarin as an indicator, on the contrary, was uniformly alkaline. The amount of acid produced, however, was very slight, amounting to from 0.3 to 0.5 c.c. of normal acid per 100 c.c. of medium. Inasmuch as these organisms undoubtedly derive their nitrogen from the nitrogen of the asparagin, it might be assumed that the acid formed was due to the removal of the basic group of the asparagin. There is a certain amount of evidence in favor of this supposition, for the organism in question would not develop in this asparagin solution without an additional source of carbon, either mannite, gelatin, or dextrose, in the experiments cited. At the same time, the breakdown of asparagin, as measured by the increase in ammonia, was so great that it would be unjustifiable to assert that these substances exert a sparing action for the nitrogen. The extent of the breakdown of asparagin was, roughly, the same in each of these media. Altho the maximum degree of nitrogen metabolism was apparently not reached even at the end of six weeks in the glycerin medium, at the end of four weeks, generally speaking, the maximum growth was reached in the other media.

It is a noteworthy fact that, even in these very simple media, the tubercle bacilli, particularly during the last three weeks of growth, were completely acid-fast. During the initial stages of growth, that is, during the first and second weeks, there was a fair proportion of non-acid-fast rods. This is in harmony with the observations made in previous experiments in which more complicated media were used. Morphologically, the organisms were indistinguishable from those

grown on the customary glycerin media, and their staining reactions, when judged by the rather crude methods available at the present time, particularly acid-fastness, were the same as those of tubercle bacilli derived from tuberculous lesions or cultures of tubercle bacilli of maximum virulence on ordinary media. In other words, the tubercle bacillus is able to build up the nitrogenous portion of its substance from asparagin, a relatively simple amino acid derivative, and its acid-fastness, be it a wax or a fat, from substances no more complex than dextrose, mannite, or glycerin, and perhaps some carbon from asparagin.

One of the noteworthy changes produced in the media by the growth of the organisms in the asparagin medium was the development of a mucinous-like substance, which was apparent even at the end of the first week. By the end of the second week it had apparently reached its maximum, altho it persisted throughout the course of the experiment. It was possible to draw out the medium in long and viscid strings by touching it with a platinum needle. Altho the medium underlying the pellicle of the tubercle bacillus exhibited this mucinous change most strongly, the organisms themselves were also somewhat mucinous in character. This viscosity was most marked in mannite, considerable in dextrose, and relatively slight in glycerin.

The relative luxuriance of growth of tubercle bacilli in this very simple medium has somewhat more than academic interest, for it has been shown by various authors that the tubercle bacillus can produce tuberculin from these compounds. Proskauer and Beck[1] showed that their cultures of tubercle bacilli would produce tuberculin in their asparagin medium, and this observation has been confirmed a number of times since. It would appear to be desirable to prepare tuberculin from a simple medium of this type, if possible, because the amount of extraneous substance is very little, consequently the reactions induced by it should be more clean-cut and less liable to misinterpretation, due to the elimination of the irritant action of peptone and other products of protein disintegration which contaminate tuberculin prepared in the usual manner.

The composition of commercial tuberculin varies greatly (White and Hollender[3]), and it is conceivable that tuberculin developed in a medium of known composition could be standardized much more accurately when it is relatively free from extraneous products than

3. Trans. National Assn. for Study and Prev. Tuberc., 1913, 9, p. 326.

when it is mixed with substances of unknown composition and of themselves irritant.

After this work was completed, an article by Möllers[4] appeared, which would seem to indicate that, in an asparagin medium containing glycerin, there is a very considerable antigen content. It is interesting to note that the maximum antigen content coincides, in time, with the greatest weight of the pellicle formed on the surface of the medium, as shown by Möllers[4] and Lockemann.[5] After reaching a maximum, both the weight of the pellicle and the antigen content, according to their observations, decrease materially in amount. Möllers concludes that the course of the weight curve of the tubercle bacillus in fluid media shows a great similarity to the curve of the antigen content of the corresponding culture fluid. It was an interesting and significant fact that the greatest weight of the pellicle of the tubercle bacillus reached a maximum and then decreased, suggesting a relationship with the formation of ammonia, which also reaches a maximum and is then followed by a recession of the ammonia content. It is conceivable that the three phenomena, the decrease in weight of the tubercle bacillus pellicle, the decrease in antigen content, and the decrease in vegetative activity, as shown by the ammonia curve, are parallel phenomena, and the cause of the decrease in each instance is closely associated with the recession of this vegetative activity.

4. Möllers. Veröffentlichgungen der Robert Koch-Stiftung zur Bekämpfung der Tuberkulose, 1914, 10, p. 56.
5. Lockemann, Ibid., p. 21.

THE METABOLISM OF CERTAIN RAPIDLY GROWING TUBERCLE BACILLI IN MEDIA WITH INORGANIC SALTS AS SOURCES OF NITROGEN

STUDIES IN ACID-FAST BACTERIA. IV

A. I. Kendall, A. A. Day, and A. W. Walker

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

It is surprising to find that an organism as complex in its activities as the tubercle bacillus should develop in a medium in which the source of nitrogen is an ammonium salt and the source of carbon an alcohol or a carbohydrate. Wherry[1] has shown that a rapidly growing strain of an avirulent tubercle bacillus will develop, with moderate luxuriance, in a medium even simpler in composition than the asparagin medium, referred to in our Study III. Wherry used ammonium chlorid as a source of nitrogen in his experiments, the various simple alcohols and carbohydrates as sources of carbon. It is essential, as he showed, to add phosphorus to these media, preferably Na_2HPO_4, where ammonium chlorid is used as a source of nitrogen, and also a certain amount of NaCl as well. Even avirulent tubercle bacilli do not grow rapidly on this medium, but the growth is unmistakable and it may be increased somewhat in luxuriance by repeated transfers. The morphology of the tubercle bacillus is considerably modified during prolonged growth on this ammonium chlorid medium, the organisms being for the most part non-acid-fast with a preponderance of coccoid elements, some of them containing acid-fast granules, the majority of the granules, however, being metachromatic.

These observations have been repeatedly confirmed in this laboratory. They are of theoretical importance; in the first place, the organisms developing under these conditions may be regarded as tubercle bacilli reduced in their chemical composition to the lowest terms compatible with growth. They contain, theoretically, but seven elements; viz., carbon, nitrogen, hydrogen, oxygen, phosphorus, sodium, and chlorin. It is conceivable that minute traces of sulphur and perhaps

1. Centralbl. f. Bakteriol., Abt. I, Orig., 1913, 70, p. 115; Jour. Infect. Dis., 1913, 13, p. 144.

other elements may be absorbed by the media from the air, but these contaminations, if they were present, were reduced to a minimum by thoroughly cleaning the glassware with which they came in contact with alkalin permanganate, oxalic and chromic acids, and water. The media were made from carefully distilled water. The cultures, furthermore, were protected during incubation by double paper caps, and they were grown in an electrically heated incubator. It would appear, therefore, if these precautions were successful, that living tubercle bacilli, containing but seven elements in their body substance, would develop in a medium containing, as a source of nitrogen, an inorganic salt, ammonium chlorid, and a simple alcohol, ethyl alcohol, as a source of carbon. These organisms, furthermore, can exist indefinitely under these conditions, for cultures in this medium have been carried on several months by repeated transfers without a diminution in the extent of the growth; indeed, there is a tendency for the growth to increase somewhat in luxuriance with repeated transfers.

The development, however, in this medium is slow, and more luxuriant growths are obtainable from the same elements using $(NH_4)_2HPO_4$ as a combined source of phosphorus and nitrogen, the other constituents remaining the same. The metabolism of a rapidly growing tubercle bacillus (597) in a medium consisting of 4 grams of $(NH_4)_2HPO_4$ and 5 grams of NaCl, dissolved in a liter of redistilled water as a basis, to which was added respectively 1 percent of dextrose, 1 percent of mannite, and 3 percent of glycerin as sources of carbon, is presented in this article. The organism was grown for several weeks in this medium, being transferred weekly before the inoculations reported on here were made. It is obvious that this procedure eliminated the possible introduction of small amounts of foreign substances at the time of inoculation and insured successful development of the organism. In experiments involving such simple media, it is obviously essential to exclude extraneous substances by a rigorous attention to the cleaning of all utensils coming in contact with this medium, the details of which have been described. This procedure may be confidently relied on to exclude extraneous contaminations.

It must be remembered that ammonia formation cannot be observed in media in which the source of nitrogen is diammonium hydrogen phosphate, because the ammonia in this compound is removed quantitatively by the Folin air current method in a strongly alkaline solution;[2]

2. Folin, Jour. Biol. Chem., 1912, 11, p. 523.

consequently, the successive determinations of nitrogen in this study are, in reality, determinations of the total nitrogen in solution in the media, and the differences observed between the initial nitrogen content and that found at various stages of growth represent the amounts of nitrogen which is locked up in the bodies of the newly formed bacteria, and perhaps also in some substance or substances formed coincidently by the bacteria which is not detected by this method. If there is such a nitrogen-containing substance or substances, which is not a part of the bacterial cell and which is not detectable by the air current method, it must at least be a synthetic product of bacterial action, for the original nitrogen content can be determined quantitatively by this method.

The tubercle bacillus, according to the analyses of various observers, contains a high percentage of phosphorus amounting to, at least, 50 per-cent of the ash of the organism,[3] and it is conceivable that some combination of phosphorus with simple amino acids or complex combinations of amino acids might be represented in this non-determinable nitrogen fraction. The experiments of Neuberg and Oertel[4] indicate that the nitrogen of these substances would not escape detection by the air current method, for these substances are labile and easily broken down in acid or alkaline media, and may even decompose on standing.

The results of such a metabolism experiment in this medium are given in Table 1.

The reaction to phenolphthalein in dextrose and mannite is uniformly alkaline; after the first week it becomes progressively acid in glycerin. Inasmuch as the media are fundamentally of the same composition, except for the source of carbon, this experiment would suggest that the organism in question produces acid incidentally to its utilization of glycerin, while it produces alkali when dextrose and mannite are the sources of carbon. This agrees with the observations of Theobald Smith on this point.[5] The reaction in dextrose is slightly acid to alizarin, in mannite it is neutral, but in glycerin it is slightly alkaline. The entire series of observations on changes in reaction to various indicators, when the same organism is grown in media varying in composition from the relatively complex nutrient broth-peptone medium to the simple ammonium phosphate glycerin medium, would suggest that these reactions are due to unknown constituents or com-

3. De Schweinitz and Dorset, Jour. Am. Chem. Soc., 1898, 20, p. 618; 1903, 25, p. 354; and 20th Annual Report of the Bureau of Animal Industry, 1903.
4. Biochem. Ztschr., 1914, 60, p. 491.
5. Jour. Med. Research, 1905, 13, p. 253.

TABLE 1

METABOLISM OF TUBERCLE BACILLUS IN MEDIA WITH INORGANIC SALTS AS SOURCES OF NITROGEN

	Dextrose					Mannite					Glycerin				
Days	Alizarin	Neutral Red	Phenol-phthalein	NH_3mg. per 100 c.c. Broth	NH_3 Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3mg. per 100 c.c. Broth	NH_3 Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3mg. per 100 c.c. Broth	NH_3 Total N_2 Percent
7	+0.10	—0.30	—0.10	—3.5	—8.34	—0.10	—0.30	—0.10	—3.5	—8.34	—0.10	—0.10	—0.10	—4.2	—10.00
14	+0.20	—0.30	—0.10	—4.2	—10.00	—0.20	—0.40	—0.20	—4.2	—10.00	—0.20	0.00	0.00	—4.2	—10.00
21	+0.30	—0.50	—0.15	—4.2	—10.00	0.00	—0.40	—0.40	—4.2	—10.00	—0.20	+0.10	+0.10	—4.9	—11.66
28	+0.20	—0.30	—0.05	—2.8	—6.56	0.00	—0.20	—0.20	—2.1	—5.00	—0.30	+0.10	+0.30	—4.2	—10.00

binations of constituents of the media, and that they are of value for the identification of these organisms only when the composition of the medium is definitely known, or, in the case of media as complex in composition as the glycerin meat juice peptone broth, where the conditions of preparations are rigorously duplicated each time. This is not to be construed, however, as indicating that these reactions under proper conditions are worthless, for, even in the complex nutrient media ordinarily used for the growth of the tubercle bacillus, these reactions appear to be definite and reliable in the hands of those who pay sufficient attention to the details of composition and preparation. The same mucinous substance, which was found abundantly in cultures of the organism grown in the asparagin medium, was found in the ammonium phosphate medium, altho somewhat less in amount. It was most marked in the mannite medium, less in dextrose, and practically absent in glycerin.

THE NITROGENOUS METABOLISM IN AMMONIUM PHOSPHATE MEDIUM

The nitrogen content of this medium was uniformly 42 mg. per 100 c.c. At the end of fourteen days the tubercle bacillus (597) had so acted on the nitrogen constituent that 10 percent of it had disappeared from the underlying culture medium; it is probable that a large percentage of this nitrogen was combined in the bodies of the organisms. A certain amount of evidence in favor of this view is afforded by the reappearance of some of this nitrogen in solution in the dextrose and mannite media on the twenty-eighth day. This experiment would suggest that the cause for the recession of the ammonia was, in some way, associated with the autolysis of the pellicle of the tubercle bacilli, which formed on the surface of the medium. There is no recession in the case of the glycerin broth. This observation is in harmony with similar observations in more complex media, where this recession of ammonia was found to be less marked when glycerin was present than when dextrose or mannite were present.

SUMMARY

A rapidly growing strain of human tubercle bacilli (597) has been grown in a medium of known and very simple composition, consisting essentially of diammonium-hydrogen-phosphate, as a combined source of nitrogen and phosphorus, and dextrose, mannite, and glycerin, respectively, as sources of carbon. At the end of two weeks, 4.2 mg.

of nitrogen, that is to say, 10 percent of the total nitrogen of the uninoculated medium, has been so changed by the growth of this organism that it cannot be recovered as ammonia. This loss of ammonia is most plausibly explained on the assumption that it has been built up into the bodies of the newly developed bacteria. At the end of four weeks, between 40 and 50 percent of this "lost" nitrogen has reappeared in the clear medium underlying the pellicle of the tubercle bacilli in such a form that it can again be determined as ammonia. The period during which the disappearance of nitrogen from the culture fluid is the greatest corresponds with the period of maximum vegetative activity in the culture. Coincidently with the reappearance of this nitrogen, which can be detected as ammonia in solution, there are evidences of a cessation of vegetative activity. This strongly suggests that the reappearance of this ammonia is associated with a certain amount of autolysis of the bodies of the bacteria.

THE METABOLISM OF "LEPRA BACILLUS," GRASS BACILLUS, AND SMEGMA BACILLUS IN PLAIN, DEXTROSE, MANNITE, AND GLYCERIN BROTHS

STUDIES IN ACID-FAST BACTERIA. V

A. I. Kendall, A. A. Day, and A. W. Walker

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

The study of the metabolism of two avirulent, rapidly growing strains of the human tubercle bacillus in plain, dextrose, mannite, and glycerin broth, respectively, showed two distinct phases in the development of the culture; an initial phase, in which the morphological picture was characterized by the development of a considerable proportion of non-acid-fast bacilli, associated with a progressive increase in the breakdown of the protein constituents of the medium, as shown by the increased formation of ammonia. This initial phase, which reached its maximum at about the third week, was succeeded by a second, recessive phase, in which all of the bacteria were acid-fast, and in which, furthermore, the ammonia which had accumulated during the initial phase gradually decreased, or at least gradually became undetectable by the method used (the Folin air current method), until finally it had practically disappeared. This same phenomenon was observed when these organisms were grown in simpler media; even in a medium as simple in composition as di-ammonium hydrogen phosphate as a combined source of nitrogen and phosphorus, with dextrose as a source of carbon, and NaCl to maintain the proper osmotic pressure of the solution.

The question arises, is this somewhat unusual series of events met with in cultures of other acid-fast bacteria, not tubercle bacilli, grown under the same conditions? If this recession of ammonia is a feature of the growth of the majority, or all acid-fast bacilli in broth, it would appear to differentiate these organisms somewhat sharply from other non-acid-fast bacteria, for the latter organisms either do not exhibit this phenomenon, or exhibit it to a lesser degree.

With this possibility in view, the metabolism of three representative types of the acid-fast group of bacteria, the grass bacillus, the smegma

TABLE 1
METABOLISM OF LEPRA BACILLUS (DUVAL)

	Mannite Broth					Glycerin Broth				
Days	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total NH_3 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent
1	0.00	—0.30	—0.10	+9.8	+3.04	—0.20	—0.10	0.00	1.4	0.44
3	+0.10	—0.20	—0.30	0.00	0.00	+0.20	+0.10	—0.10	1.4	0.44
10	—0.20	—0.30	—0.80	—4.9	—1.52	—0.40	—0.10	—0.50	- -0.7	—0.22
21	—0.10	—0.50	—0.90	—4.9	—1.52	—1.80	—1.00	—0.80	—5.6	—1.74
28	—0.40	—0.80	—0.60	—9.8	—3.04	—1.70	—1.10	—1.30	—9.1	—2.82
43	—1.20	—1.80	—1.40	—6.3	—1.95	—1.30	—1.40	—1.30	—11.2	—3.48
51	......	......	......	......	......	—1.60	—1.40	—1.70	—13.3	—4.13

TABLE 2
METABOLISM OF GRASS BACILLUS III.

	Plain Broth					Dextrose Broth				
Days	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent
1	—0.10	—0.10	—0.10	—0.7	—0.22	—0.90	—0.20	—0.70	9.8	3.04
3	—0.30	—0.40	—0.20	1.4	0.44	—0.80	—0.30	—0.90	—0.7	—0.22
6	—1.00	—0.40	—1.10	4.2	1.30	—2.20	—0.90	—1.80	—0.7	—0.22
10	—2.70	—1.40	—1.70	14.7	4.57	—2.90	—1.20	—1.60	5.6	1.74
15	—1.90	—2.10	—1.50	24.5	7.60	—3.50	—2.80	—2.00	23.8	7.40
21	—3.60	—4.00	—2.00	23.8	7.40	—3.50	—2.00	—2.20	21.7	6.75
28	—2.50	—2.00	—1.60	20.3	6.30	—3.50	—2.80	—2.20	16.1	5.00
36	—3.10	—1.30	—1.90	7.0	2.2	—2.80	—2.20	—2.30	8.4	2.60
43	—2.20	—2.10	—1.80	1.4	0.44	—2.50	—2.10	—2.40	1.4	0.44
52	—1.90	—2.00	—2.00	—4.9	—1.52	—2.30	—1.80	—2.10	—4.9	—1.52

TABLE 3
METABOLISM OF SMEGMA BACILLUS

	Mannite Broth					Glycerin Broth				
Days	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent
1	—0.30	—0.30	—0.30	8.4	2.60	—0.60	—0.20	—0.30	—0.7	—0.22
3	—1.00	—0.50	—0.70	0.00	0.00	—0.10	0.00	—0.20	—0.7	—0.22
6	—1.70	—0.70	—1.30	0.00	0.00	—1.00	—0.40	—0.50	+4.2	1.30
10	—1.40	—0.80	—0.80	1.4	0.44	+0.10	—0.70	—0.30	—2.8	—0.87
15	—1.30	—1.60	—0.50	16.8	5.22	—0.70	—0.40	—0.50	—2.8	0.87
21	—1.50	—1.20	—0.90	16.8	5.22	+1.20	+1.60	+0.60	+12.6	+3.91
28	—2.90	—2.30	—1.50	28.0	8.70	+0.70	+1.10	+0.60	14.0	4.40
36	—2.20	—2.20	—1.80	14.0	4.35	—0.40	—0.80	—0.60	0.7	0.22
43	—2.40	—2.10	—1.60	19.6	6.04	+0.70	—0.60	0.00	0.7	0.22
52	—1.50	—2.20	—2.00	—5.6	—1.74	—0.30	—0.80	—0.40	—4.9	—1.52

TABLE 1
METABOLISM OF LEPRA BACILLUS (DUVAL)—(*Continued*)

	Plain Broth					Dextrose Broth				
Days	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent
1	0.00	—0.10	0.00	0.00	0.00	—0.70	—0.30	—0.50	—0.7	—0.22
3	+0.10	0.00	—0.20	0.00	0.00	—0.10	0.00	—0.60	—1.4	—0.44
10	—1.30	—0.60	—1.20	0.00	0.00	—1.30	—0.60	—1.20	0.00	0.00
21	—0.10	—0.40	—0.70	0.7	0.22	—2.00	—1.10	—1.00	—1.4	—0.44
28	—2.30	—1.50	—1.50	—2.8	—0.87	—2.00	—1.00	—0.90	—12.6	—3.92
43	—2.30	—1.90	—2.20	—12.6	—3.92	—2.10	—1.50	—2.20	—16.8	—5.20
51	—1.40	—1.40	—1.90	—11.9	—3.70	—1.50	—1.60	—2.20	—14.7	—4.56

TABLE 2—(*Continued*)
METABOLISM OF GRASS BACILLUS III

	Mannite Broth					Glycerin Broth				
Days	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent
1	+0.10	—0.20	—0.40	9.8	3.04	—0.40	—0.10	—0.10	7.7	2.39
3	+0.10	—0.30	—0.40	0.00	0.00	—0.70	—0.20	—0.30	0.00	0.00
6	—0.10	—0.30	—0.70	0.7	0.22	—1.20	—0.50	—1.00	0.00	0.00
10	—1.10	—0.50	—0.90	1.4	0.44	—0.70	—0.30	—0.40	7.7	2.39
15	—1.50	—1.50	—1.10	8.4	2.60	—0.90	—1.20	—0.30	13.3	4.13
21	—1.50	—1.30	—1.30	15.4	4.78	—0.20	—0.50	—0.30	16.8	5.22
28	—2.10	—1.90	—1.30	11.9	3.70	—1.10	—0.90	+0.30	13.3	4.13
36	—1.90	—1.70	—1.40	16.1	5.00	—0.50	—1.40	+0.10	15.4	4.78
43	—1.80	—1.90	—1.80	2.8	0.88	0.00	—1.20	0.00	4.9	1.52
52	—1.60	—1.70	—1.80	—4.9	—1.52	—0.50	—0.60	—0.20	1.4	0.43

TABLE 3
METABOLISM OF SMEGMA BACILLUS—(*Continued*)

	Plain Broth					Dextrose Broth				
Days	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent	Alizarin	Neutral Red	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 / Total N_2 Percent
1	—0.80	—0.40	—0.50	9.1	2.83	—1.50	—0.40	—0.90	8.4	2.61
3	—1.20	—0.50	—1.10	0.7	0.22	—1.20	—0.60	—1.10	—0.7	—0.22
6	......	......	......	......	......	—1.90	—1.00	—1.90	5.6	1.74
10	—2.20	—1.30	—1.50	11.2	3.48	—2.90	—1.50	—1.10	18.9	5.87
15	—3.60	—2.20	—1.50	19.6	6.08	—3.00	—2.30	—1.10	22.4	6.96
21	—3.40	—1.20	—1.60	7.7	2.39	—4.10	—1.80	—2.10	28.0	8.70
28	—1.50	—1.60	—1.40	7.7	2.39	—3.50	—2.80	—2.10	20.3	6.30
36	—0.70	—1.20	—1.30	0.00	0.00	—3.90	—2.60	—1.20	23.8	7.40
43	—1.70	—1.80	—1.80	—4.2	—1.30	—3.30	—2.40	—2.40	18.2	5.66
52	......	......	......	......	......	—2.00	—2.20	—2.20	—7.7	—2.39

bacillus, and the "lepra bacillus" isolated by Duval, were studied in plain, dextrose, mannite, and glycerin nutrient broths. The technic of experimentation throughout was that used for the study of the tubercle bacillus.

The results, shown in tabular form, are self-explanatory in the light of the observations made on the rapidly growing, human tubercle bacilli. The smegma bacillus and the grass bacillus, altho they do not form as much ammonia under parallel conditions as did the tubercle bacilli, present a well-marked maximum followed by a steady decline in the amount of ammonia detectable in the media in which they were grown. The "lepra bacillus" appears to be somewhat different from the grass and smegma bacilli. First, in that the amount of ammonia produced is very slight, the maximum, 1.40 mg. per 100 c.c. broth, being found in the mannite medium at the end of twenty-four hours' incubation. In all media there was actually less ammonia after a few days' incubation than there was in the uninoculated control. This observation agrees with one recorded previously,[1] where, however, the experiment was only carried on for nine days. The cultural reactions and the curve of metabolism of this bacillus would seem to distinguish it rather sharply from the two organisms mentioned above.

SUMMARY

The metabolism of the smegma and grass bacilli resembles that of the rapidly growing, human tubercle bacilli, described previously, in two important particulars; neither dextrose, mannite, nor glycerin exhibits any appreciable sparing action for the protein constituents of the broth, the amounts of ammonia produced being practically the same in these media as in plain broth; and their cultures present a gradual increase in proteolysis to a maximum which is followed by a clearly defined recession of the metabolism indicated by a gradual decrease in the ammonia content.

The "lepra bacillus" does not present this metabolic phenomenon. This would suggest that this bacillus was entirely distinct in its cultural relationships from the grass and smegma bacilli, which follow more closely the metabolism of the tubercle bacillus.

1. Kendall, Day and Walker, Jour. Am. Chem. Soc., 1913, 35, p. 1248.

THE OCCURRENCE OF A SOLUBLE LIPASE IN BROTH CULTURES OF TUBERCLE BACILLI AND OTHER ACID-FAST BACTERIA

STUDIES IN ACID-FAST BACTERIA. VI

A. I. Kendall, A. W. Walker, and A. A. Day

(*From the Department of Bacteriology, Northwestern University Medical School, Chicago.*)

The growth of two rapidly growing strains of human tubercle bacilli in media of varied composition showed consistently two distinct phases in their metabolism: an initial phase characterized by rapid vegetative activity and progressive proteolysis, as indicated by a steady increase in ammonia formation; and a second phase marked by a progressive decrease in vegetative activity, and by recessive changes in metabolism, during which the ammonia accumulated during the initial phase gradually disappeared from the culture media (These Studies, I-V).

This second phase, which appears to begin rather regularly after three weeks' growth in ordinary media with the organisms studied, is of unknown causation. It is quite probable that the accumulation of waste products plays a prominent part in restricting the activity of the organisms, but the simple restriction of bacterial growth *per se* does not explain the associated phenomenon of the recession of the ammonia which has been produced during the period of rapid development. Furthermore, attempts to correlate the recession of ammonia with the presence of certain substances in the media, in which these observations were made, were unsuccessful, for even in a culture solution as simple in composition as $(NH_4)_2HPO_4$, NaCl, and dextrose, the same phenomenon was observed, suggesting strongly that the explanation of the recession is to be sought for in the organisms themselves. This at once focuses attention on the possibility of ferments, exo- or endo-cellular, being concerned, the entire process, perhaps, being one in which autolysis plays a prominent part.

The tubercle bacillus and other acid-fast bacteria are notoriously resistant to solvents which will promptly destroy other organisms not acid-fast. In the animal body, similarly, these bacteria are resistant to

lytic powers which usually suffice to destroy corresponding numbers of the ordinary organisms. Fats and waxes, which are present in considerable amount in the substance of these acid-fast bacteria, appear to confer this resistance to solution on these organisms. Consequently, the removal or modification in composition of these substances would appear to be an essential factor in the autolysis of the acid-fast type of bacteria.

Comparatively little appears to have been written on the subject of fat-splitting by bacterial ferments. It is claimed by Rubner[1] and others that the microbic decomposition of fats with the liberation of fatty acids and glycerin is in reality a "fat fermentation" brought about by the direct activity of the protoplasm of the organism, not to a definite fat-splitting ferment elaborated by them. On the other hand, Sommaruga[2] and others appear to have demonstrated lipases in several types of bacteria; Carrière[3] demonstrated a lipase in the bodies of six-months-old tubercle bacilli, but not in the media in which they were grown; Wells and Corper[4] have also demonstrated lipases in cultures of tubercle bacilli.

In order to determine whether esterases or lipases play a part in the development of the tubercle bacillus in artificial media, a series of cultures of various strains of acid-fast bacilli of various ages, including not only human tubercle bacilli of various degrees of virulence, but also bovine and avian tubercle bacilli as well, and, in addition, certain other acid-fast bacteria, including the grass bacillus, the leprosy bacillus isolated by Duval, and the smegma bacillus, were examined for evidence of ester- and fat-splitting ferments. The organisms investigated, without exception, grew as a firm, tenacious pellicle on the surface of the medium, leaving the underlying fluid perfectly clear. This clear fluid, free from bacteria, was used in these experiments.

The technic adopted was as follows: One cubic centimeter of this clear fluid underlying the bacteria was placed in a large, clean test-tube with 10 c.c. of freshly distilled water, using sterile precautions; 0.25 c.c. of ethyl butyrate (neutral in reaction) and 0.5 c.c. of toluene were then added, and the whole shaken one hundred times in order to produce a well-mixed emulsion.[5] Two drops of phenolphthalein were then added and the mixture brought to the neutral point by titration with N/50 acid or alkali, depending on the reaction. This mixture was then stoppered and incubated at 37° C. for twenty-four hours, and again brought to neutrality with N/50 alkali. The increase in acid-

1. Arch. f. Hyg., 1900, 38, p. 67.
2. Ztschr. f. Hyg. u. Infektionskrankh., 1890, 18, p. 441.
3. Compt. rend. Soc. biol., 1901, 53, p. 320.
4. Jour. Infect. Dis., 1912, 11, p. 388.
5. Jobling and Bull. Jour. Exp. Med., 1912, 16, p. 483.

ity, measured in terms of N/50 NaOH, was taken as a measure of the lipolytic activity of the culture.

All of the cultures tested for lipase in this series were grown in broth, uniform in composition, in volume (100 c.c.), and with the same area of free surface. It will be seen that the increase in acidity observed represents, theoretically, 1/100 of the total lipolytic activity of the particular culture under discussion, or, better, 1/100 of the amount of acid which would be liberated by the entire culture in twenty-four hours under the given conditions, for it will be remembered that but 1 c.c. of the culture was used for the test: the results, therefore, are comparative, and roughly quantitative.

Almost without exception, the culture media in which these acid-fast bacteria had grown were rather dark-colored, and this color was sufficient in undiluted solutions to obscure somewhat the end point to phenolphthalein during titration. For this reason 1 c.c. of the culture medium was diluted with 10 c.c. of freshly boiled, distilled water. This dilution accomplished three results: boiling the water drove out all CO_2, and the re-entrance of CO_2 was prevented by the layer of toluene which formed a seal on the surface; diluting the broth with 10 c.c. of water also diluted the color proportionately, so that the end point was very much more distinct; finally, the dilution also prevented any considerable concentration of products of ferment activity which might otherwise tend to arrest the process.

It was found by experiment that 0.25 c.c. of ethyl butyrate was sufficient to react with all the lipase present in the media. This ethyl butyrate was freed from acid before it was added to the media.

Toluene was added for two reasons: first, to prevent the absorption of CO_2 from the air and thus increase the acidity of the medium; and, secondly, to restrain the development of any extraneous bacteria which might have inadvertently reached the solution during the process of preparation and titration: 0.5 c.c. of toluene was sufficient to accomplish these two results.

When the diluted broth, ethyl butyrate, and toluene were prepared in the manner indicated above, the tube in which they were placed was agitated thoroughly in order to mix the substances as intimately as possible.

As a rule, the initial reaction of the lipase solution thus prepared was approximately normal to phenolphthalein at the start, but occasionally cultures were found in which there was a slight initial acid or alkaline reaction. In every instance, the reaction was adjusted to a faint pink by the addition of N/50 acid or alkali, as the case might be, prior to incubation.

At the end of twenty-four hours' incubation at 37 C. the tubes were again shaken and the reaction determined by titration with N/50 NaOH. Incubation was continued for seventy-two hours in the early determinations to measure the total ester-splitting power of the media, but the increase in acidity occasionally detectable after twenty-four hours was found to be so slight it was disregarded in these tables, which are designed to show the presence, rather than the exact measure, of the lipase produced by these organisms. Indeed, duplicate determinations on the same sample indicate that the inherent errors of the method (indicator error and error of sampling) practically compensated for these very slight increases which were occasionally detected after seventy-two hours' incubation. The average difference between duplicate determinations is about 0.1 c.c. N/50 NaOH, and this figure appears to represent the average error of the method. The increase in acidity in cubic centimeters of this solution was taken as a measure of the activity of the lipase in the culture medium. It should again be remarked parenthetically that the theoretical lipase activity of the entire culture would be one hundred

times the amount determined in the process described above, for but 1/100 of the medium was used for this determination.

Throughout these experiments, controls were made in the following manner. A media control in which 1 c.c. of uninoculated (sterile) media of the same composition as that of the cultures was prepared in precisely the manner outlined above, the only difference being that the media control was free from any products of bacterial development. A second media control was made in which the broth containing the lipase was incubated under parallel conditions without the ester. This latter control was designed to show any changes in reaction which might take place spontaneously in the medium in the absence of the ester. A third ester control was used in which the ester was prepared with toluene and distilled water, but to which no broth, either inoculated or uninoculated, was added. These controls were incubated under precisely the same conditions as the lipase solutions, and, without exception, these controls have invariably been neutral at the end of twenty-four hours: that is to say, there was absolutely no increase in acidity in these controls which contained uninoculated broth and an ester, inoculated broth and no ester, and an ester alone. Consequently, the accumulation of acid observed in these experiments after twenty-four hours is attributable to the splitting of the ethyl butyrate by a reactive substance developed in these media during the growth of the organisms, resulting in the liberation of butyric acid. From the conditions of the experiment it would appear that this substance, which split the butyric ether, was a fat-splitting ferment. It might be objected that a ferment which splits ethyl butyrate should be termed an esterase and not a lipase: such esterases, which do not decompose complex glycerids, occur in the human body. Achard and Clerc, and Saxl[6] have shown that an esterase which splits monobutyrin is present in the blood serum of man; they were, however, unable to demonstrate the presence of a true lipase. In order to answer this objection, the same series of experiments was carried out, using castor oil in place of ethyl butyrate as a substrate. The castor oil was first neutralized and emulsified by the cautious addition of N/10 NaOH until a faint permanent pink reaction resulted, according to the method of Kanitz.[7] This emulsion remained permanent for several days: 0.5 c.c. of this emulsion was added to a series of tubes prepared precisely as those described above, with the same controls.

Culture "597" is a rapidly growing human tubercle bacillus which was obtained from the American Museum of Natural History of New York through the kindness of Professor Winslow. Culture "W" is an avirulent, rapidly growing, human tubercle bacillus which came from the laboratory of Professor Wherry of the University of Cincinnati. Culture "257" is also a human tubercle bacillus intermediate in its growth and virulence between the rapidly growing strains just described and cultures human and human "X," which were obtained from Prof. Theobald Smith of the Harvard University Medical School. These latter cultures, human and human "X," are experimentally pathogenic for guinea-pigs. The bovine tubercle bacillus came originally from the Bureau of Animal Industry, as did the avian culture. For these last two organisms we are indebted to Dr. Enos Day, Director of the Pathological Laboratory at the Chicago Stock Yards. The leprosy bacillus was one isolated by Duval. The smegna bacillus and Grass bacillus III were cultures the histories of which are unknown to us.

6. Achard et Clerc, Compt. rend Soc. de biol., 1902, 54, p. 1144. Saxl, Biochem. Ztschr., 1908, 12, p. 343.
7. Ztschr. f. physiol. Chem., 1905, 46, p. 482.

Table 1 shows the organism, the medium in which the organism was grown, the age of the culture, and the amount of acid in terms of N/50 NaOH, which developed when 1 c.c. of each of these cultures, respectively, was incubated twenty-four hours under the conditions outlined above. It will be observed that, as a general rule, the degree of acidity was somewhat greater when ethyl butyrate was used as a substrate than when castor oil was used for the same purpose, under the same conditions. While the amounts of acid produced by the bacteria shown in this table are not large, it must be remembered that the theoretical acidity, which would be developed by the entire culture under parallel conditions, would have been 100 times the values given. It would appear, therefore, that, without exception, these acid-fast

TABLE 1
Amount of Acid Produced

Organism	Medium	Age of Culture	C.C. N/50 NaOH	
			Ethyl Butyrate	Castor Oil
B. tuberculosis-Human 597..	Mannite broth	3 months	2.10	1.85
B. tuberculosis-Human W ..	Glycerin broth	4 months	1.25	1.15
B. tuberculosis-Human X...	Glycerin broth	4 months	1.35	1.20
B. tuberculosis-Human	Glycerin broth	4 months	1.80	1.65
B. tuberculosis-Human 257..	Glycerin broth	1 month	1.30	1.35
B. tuberculosis-Avian	Glycerin broth	1 month	1.25	1.35
B. tuberculosis-Bovine	Glycerin broth	2 months	1.00	0.95
B. leprae (Duval)	Glycerin broth	1 month	0.35	0.20
B. smegmatis	Glycerin broth	1 month	1.00	0.90
B. grass IV	Glycerin broth	1 month	1.20	1.15
Control (media)	Glycerin broth	—	0.00	0.00
Control (ester)	—	—	0.00	0.00

organisms produce, as the result of their growth in artificial media, a substance which is in solution and which possesses the property of splitting both ethyl butyrate and castor oil. From the nature of this splitting it would appear justifiable to designate this substance a lipase.

In order to throw some light on the physical properties of this lipase, a series of experiments was made with the same organisms, using different cultures, however, to determine the degree of resistance of this lipase to heat. A series of initial experiments was made in which it was found that heating to 60 C. for thirty minutes had little or no effect on the activity of the lipase. A second series of experiments was made in which 1 c.c. of the respective culture fluids, suspended in 10 c.c. of distilled water, was heated at the temperature of boiling water for fifteen minutes, and then, after the addition of the

ester and toluene, compared under precisely the same conditions with unheated cultures of the same bacteria. It will be seen from Table 2 that even heating to 100 C. for fifteen minutes is without noteworthy effect on the activity of this lipase. At first sight, this unexpected resistance of the reactive substance to heat would seem definitely to eliminate a ferment as the cause of this splitting of the ester and castor oil, for the vast majority of ester- and fat-splitting ferments which have been studied up to the present time have been found to be thermolabile, losing their activity at temperatures above 60 C. Carrière found that the lipase of the tubercle bacilli, which he studied, was thermolabile. Loevenhart,[8] however, has shown that the lipase of the pancreas, when dried, can be heated at 110 C. for one and one-half hours with the loss of about 50 percent of its activity when measured on ethyl butyrate, altho its action on olive oil remained undiminished. Abbott and Gildersleeve[9] have shown that certain proteolytic enzymes of bacterial origin may be heated in the moist state to a temperature of 100 C. for fifteen to thirty minutes without impairing their characteristic function. Other observers also have found that ferments of bacterial origin may be thermostabile. With these observations in mind it appears logical to attribute the splitting of ethyl butyrate and castor oil observed above to the action of a true lipase.

TABLE 2
Effect of Heat on the Lipase

Organism	Medium	Age of Culture, Days	C.C. N/50 NaOH Ethyl Butyrate	
			Unheated	Heated
B. tuberculosis W..........	Plain broth	21	1.40	1.35
B. tuberculosis W..........	Dextrose broth	21	1.40	1.30
B. tuberculosis W..........	Mannite broth	21	1.45	1.25
B. tuberculosis W..........	Glycerin broth	21	1.35	1.30
B. leprae (Duval)	Plain broth	21	—0.15	—0.25
B. leprae (Duval)	Dextrose broth	21	—0.20	—0.20
B. leprae (Duval)	Mannite broth	21	—0.15	—0.20
B. leprae (Duval)	Glycerin broth	21	—0.10	—0.25
B. smegmatis	Plain broth	21	1.20	1.20
B. smegmatis	Dextrose broth	21	1.20	0.80
B. smegmatis	Mannite broth	21	1.40	1.15
B. smegmatis	Glycerin broth	21	1.50	1.20
B. grass IV	Plain broth	21	1.30	1.10
B. grass IV	Dextrose broth	21	1.25	—
B. grass IV	Mannite broth	21	1.10	1.40
B. grass IV	Glycerin broth	21	1.20	1.30

8. Jour. Biol. Chem., 1907, 2, p. 451.
9. Jour. Med. Research, 1903, 10, p. 61.

Most ferments are said to be non-diffusible. In order to test the diffusibility of the lipase found in the cultures of acid-fast bacilli, two distinct methods were tried: diffusion through agar and diffusion through collodion membranes.

To determine the diffusibility of the lipase through agar, a series of test-tubes of uniform diameter (1.5 cm.) and length (15 cm.) were bent at right angles midway of the long axis, so that the resulting formation resembled a capital "L." Each test-tube was then filled to about two-thirds of its length with glycerin agar, slanting the agar in that portion of the tube nearer the open end, and then inoculating the slanted surface with one of the rapidly growing human tubercle bacilli. In this way a maximum surface growth of the organism was obtained. These cultures were incubated and examined at regular intervals as follows: One of the bent agar tubes was broken in such a manner that the agar in the closed end was removed absolutely free from the growth on the slanted surface, thus eliminating bacteria. This agar was divided into two parts, A and B; A being nearer the slanted surface, B being more remote from the slanted surface. These portions of agar were then macerated with sterile precautions in 5 c.c. of sterile distilled water, 0.25 c.c. of ethyl butyrate added together with 0.5 c.c. of toluene, shaken, neutralized, and the whole then incubated at 37 C. for twenty-four hours. If any lipase had diffused from the bodies of bacteria on the slanted surface of the agar through to the closed arm from which these samples were taken, the customary splitting of the ethyl butyrate and castor oil should take place. A series of twelve such determinations was made, growths from one week to three months being examined in the series, with absolutely uniform negative results; that is to say, this experiment would indicate that an active lipase had not diffused in demonstrable amounts into and through the agar. The organisms grown on the surface, however, contained lipase in every instance. This experiment was not deemed conclusive, for it is conceivable that the composition and reaction of the agar may have inhibited the action of the ferment.

Another attempt was made to determine the diffusibility of the lipase by suspending a collodion sac, 1 cm. in diameter and 10 cm. long, filled with broth known to contain an active lipase, in a test-tube 2 cm. in diameter, the space between the collodion sac and the walls of the tube being occupied by sterile distilled water, with a layer of toluene overlying both the contents of the sac and the water outside the sac.

Even after a week's incubation at 37 C., the fluid outside the collodion membrane being tested daily after addition of ethyl butyrate in the usual manner, no evidence of lipase activity could be detected outside the collodion sac. The fluid within the collodion membrane showed uniformly strong lipolytic action both on ethyl butyrate and castor oil. This experiment was repeated in detail, using the bodies of tubercle bacilli, which had been washed thoroughly and then killed with toluene water, in place of the broth culture containing the lipase; the supposition being that, as the tubercle baccilli autolyzed, a certain amount of lipase might escape from them and diffuse through the collodion membrane into the surrounding distilled water. Here again the results were uniformly negative, altho lipase of considerable strength could be demonstrated in the fluid inside the collodion membrane, where, it will be remembered, the tubercle bacilli were undergoing autolysis.

CONCLUSIONS

The evidence indicates that a variety of acid-fast bacteria, including various strains of the human tubercle bacillus, the bovine, and avian tubercle bacilli, as well as the leprosy, smegma, and grass bacilli, form lipase during their growth in glycerin broth.

The lipase is present in the medium free from the bacteria.

The lipase resists an exposure of fifteen minutes to 100 C. in the moist state without appreciable diminution in its activity.

The lipase formed by these organisms appears to be non-diffusible, at least in an active state, through either agar or a collodion membrane.

THE RELATIVE ACTIVITY OF THE SOLUBLE LIPASE AND LIPASE LIBERATED DURING AUTOLYSIS OF CERTAIN RAPIDLY GROWING TUBERCLE BACILLI

STUDIES IN ACID-FAST BACTERIA. VII

A. I. KENDALL, A. W. WALKER, AND A. A. DAY

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

In the previous article,[1] experiments were recorded which demonstrated that an active fat-splitting ferment was present in solution in broth cultures of a variety of acid-fast bacilli. These experiments were carried out in such a manner as to be roughly quantitative, and the activity of the lipase, as determined by these experiments, was found to be very considerable. It is a matter of importance to determine whether these lipases are excreted by these acid-fast bacilli as exo-ferments, or whether they are bound up in the bodies of the bacteria, and appear in solution only when the bacteria have autolyzed (endo-ferments). Carrière[2] has described a thermolabile lipase in the organisms of a six-months-old culture of the tubercle bacillus; his observations suggest that this lipase was an endo-ferment and that even minute amounts of culture medium destroy or inactivate it. This observation is not in harmony with the results presented above, for the lipase of the acid-fast bacteria described there was present and active in the culture medium, and it was thermostabile. It is possible, however, that the lipase, which is demonstrable in younger cultures of tubercle bacilli, may have disappeared, as the cultures were incubated for a longer period, without affecting the activity of a second thermolabile endo-lipase which is locked up within the bacteria themselves.

The following observations were undertaken with a view of determining the relative amounts of lipase, respectively, in the culture medium and in the bodies of the bacteria grown in the culture medium. The cultures (various acid-fast bacilli) were of unequal ages, as indicated in Table 1. Various degrees of autolysis of the bacteria, therefore, must have taken place, depending on the strain of organism studied and the length of incubation.

1. Study VI.
2. Compt. rend. Soc. de biol., 1901, 53, p. 320.

The plan of procedure was as follows: For the determination of the lipase in solution in culture media, the method described in the previous article[3] was followed exactly. In order to determine the activity of the lipase present in the organisms themselves (which form a tenacious pellicle on the surface of the media), the bacteria were first separated quantitatively from the culture medium and washed free of adherent soluble lipase by filtration through filter paper, then washed three successive times with redistilled water, each time with 100 c.c.

The organisms, freed from all adherent culture medium by this thorough washing, were suspended in 10 c.c. of redistilled, freshly boiled water containing toluene to kill them, then they were mixed intimately with 1 c.c. of ethyl butyrate and 1 c.c. of castor oil, respectively; and again shaken after the further addition of 0.5 c.c. of toluene. A second set of experiments, using parallel cultures of the same bacteria was prepared, using castor oil in place

TABLE 1
AMOUNT OF LIPASE IN MEDIUM AND IN BODIES OF BACTERIA

Organisms	Medium	Age	Cc. N/50 NaOH			
			Filtrate		Bacteria	
			Ethyl Butyrate	Castor Oil	Ethyl Butyrate	Castor Oil
B. tuberculosis W	Glycerin	3 months	1.25	1.00	4.05	3.80
B. tuberculosis 597	Mannite	2 months	1.10	0.60	2.30	2.10
B. tuberculosis-Human X	Glycerin	3 months	1.35	1.20	3.30	2.25
B. tuberculosis-Human	Glycerin	3 months	1.00	0.90	2.30	1.70
B. tuberculosis-Human 257	Glycerin	1 month	1.30	0.95	3.35	2.55
B. tuberculosis-Avian	Glycerin	1 month	1.60	1.40	3.70	2.90
B. tuberculosis-Bovine	Glycerin	3 months	1.95	1.40	3.55	2.45
B. leprae (Duval)	Glycerin	3 weeks	0.65	—	4.10	—
B. grass III	Glycerin	3 weeks	1.60	—	5.60	—
B. smegmatis	Glycerin	3 weeks	1.55	—	3.55	—
Control	Glycerin	—	0.00	0.00	0.00	0.00
Control	—	—	0.00	0.00	0.00	0.00

of ethyl butyrate as the substrate. The filtrates of these parallel cultures gave practically identical results, and, for the sake of brevity, but one set of results is reported under "filtrates," as shown in Table 1. The suspensions containing the bacteria were then neutralized to phenolphthalein, using N/50 acid or alkali, as the case demanded. It should be remarked parenthetically that, without exception, the reaction of the organisms after this washing was practically neutral, so that one or, at most, two drops of N/50 acid or alkali were required to bring the reaction to the neutral point of phenolphthalein. The bacteria and the filtrates, prepared according to the procedure outlined, with suitable media and ester controls, were then incubated at 37 C. for twenty-four hours, and the increase in acidity determined by titration with N/50 NaOH. The suspensions of bacteria were further titrated for three successive days, at the end of which time the activity of the lipase was greatly diminished.

3. Study VI.

Table 1 shows the lipase content, respectively, of the broth and the bacteria in terms of cubic centimeters of N/50 alkali. It will be observed that the determination of lipase in the broth was not carried beyond the twenty-fourth hour of incubation; with the bacteria, however, daily titrations were made, the observations being prolonged for three days. The amount of lipase in the filtrate was found to be between 0.65 and 2.00 c.c. of N/50 acid, but it must be remembered that the total lipase content of the 100 c.c. of the broth, from which the bacteria were derived, would be one hundred times this amount, theoretically. The amount of lipolytic activity exhibited by these organisms, while considerable in amount, is noticeably less than the total theo-

TABLE 2

Amount of Lipase in Washings and in Bacteria Autolyzed after Washing

Organisms	Medium	Age	Cc. N/50 NaOH to Neutralize Ethyl Butyrate							
			24 Hours Incubation				Bacteria Autolyzed after Washing			
			Filtrate from Bacteria	Washings			Incubation			
				A	B	C	24 Hrs.	48 Hrs.	72 Hrs.	Total
B. tuberculosis W	Plain broth	6 weeks	0.65	0.05	0.00	0.00	2.35	2.30	0.30	4.95
B. tuberculosis W	Dextrose broth	6 weeks	1.30	0.10	0.00	0.00	2.80	2.90	0.75	6.45
B. tuberculosis W	Mannite broth	6 weeks	1.15	0.05	0.00	0.00	2.35	2.20	0.20	4.75
B. tuberculosis W	Glycerin broth	6 weeks	1.75	0.05	0.00	0.00	2.30	1.60	0.30	4.20
B. tuberculosis 597	Plain broth	6 weeks	0.45	0.05	0.00	0.00	2.20	2.15	0.50	4.85
B. tuberculosis 597	Dextrose broth	6 weeks	1.20	0.10	0.00	0.00	2.85	3.30	0.15	6.30
B. tuberculosis 597	Glycerin broth	6 weeks	1.70	0.20	0.00	0.00	3.15	2.25	0.60	6.00
Control	Medium and ester	—	0.00	....	....	....	....	....	....	....
Control	Wash'gs and ester	......	....	0.00	0.00	0.00	....	....	....	....
Control	Ester	......	0.00	....	....	....	0.00	....	....	....

retical fat-splitting power of the underlying culture medium. The cultures had been incubated for the most part from one to three months, so that it would be justifiable to assume considerable autolysis before these determinations were made. Nevertheless, the very considerable disparity observed between the relatively large amount of lipolytic activity of the filtrates on the one hand, and of the bacteria which grew in these culture media on the other, would suggest the possibility that the ferment in the culture medium, in part at least, was an extracellular lipase, unless it be assumed that this lipase was an endo-ferment which had escaped from the substance of the bacteria into the underlying medium during their autolysis prior to these experiments. The experiment does not determine this point.

In order to determine the amount of lipase lost in washing these bacteria and to throw some light on the possible influence of the source of carbon on the production of lipase, an experiment was made as follows: two rapidly growing human tubercle bacilli, "W" and "597," were grown for six weeks in plain, dextrose, mannite, and glycerin broths, respectively. At the end of that time the organisms were separated quantitatively from the underlying medium by filtration through filter paper, then they were washed with three successive portions of distilled water. The washings were labelled A, B, and C, respectively. The determinations were made as indicated in Table 2.

The results indicate that the filtrates from plain broth cultures were uniformly less active lipolytically than the filtrates of the same organisms grown in dextrose, mannite, and glycerin broths. There was very little lipase in the first washing (A), none in the second and third washings (B and C). The results otherwise are self-explanatory.

CONCLUSIONS

The observations appear to justify the conclusion that certain acid-fast bacteria grown in nutrient broth, with dextrose, mannite, and glycerin as additional sources of carbon, produce an active lipase which appears in solution in the various culture media.

The bodies of the bacteria, freed from adherent culture media and soluble lipase by thorough washing, also contain an active lipase, probably liberated as the bacteria underwent autolysis.

The lipase in solution appears to be either greater in amount, or more active than that contained in the bacteria freed from the culture medium.

It cannot be stated whether the lipase free in the culture media is freed as the result of autolysis of the bacterial cells (endo-lipase), or whether it is secreted by the bacteria as an exo-lipase.

OBSERVATIONS ON THE SPECIFICITY AND THERMOSTABILITY OF THE LIPASE DEVELOPED DURING THE GROWTH OF A RAPIDLY GROWING TUBERCLE BACILLUS IN MEDIA OF VARIED COMPOSITION

STUDIES IN ACID-FAST BACTERIA. VIII.

A. I. KENDALL,
A. W. WALKER,
AND
A. A. DAY

Reprinted from
The Journal of Infectious Diseases, Vol. 15, No. 3, Nov. 1914, pp. 455-459

OBSERVATIONS ON THE SPECIFICITY AND THERMOSTABILITY OF THE LIPASE DEVELOPED DURING THE GROWTH OF A RAPIDLY GROWING TUBERCLE BACILLUS IN MEDIA OF VARIED COMPOSITION

STUDIES IN ACID-FAST BACTERIA. VIII

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

A. I. Kendall, A. W. Walker, and A. A. Day

The constant presence of a thermostabile lipase, both in the filtrates of broth cultures of certain acid-fast bacilli (including human, bovine, and avian tubercle bacilli, and others) and in the organisms themselves,[1] focuses attention on the possible identity of the lipase produced by these various bacteria. Before this question can be answered, it is desirable to study the effect of the composition of the medium on the nature and extent of the lipolytic activity manifested by a specific organism; for it is conceivable that this lipase may be present in all media in which the given organism will grow, irrespective of composition, or, on the other hand, it may be developed only in the presence of certain substances contained in these media, presumably those on which it can act.

In the first instance, the lipase would be an integral factor in the life history of the organism, whereas in the second case the production of a lipase might be regarded as a definite response on the part of the bacteria to the presence of a specific substance, or substances, in the substrata on which the organisms have grown. If the first assumption is correct, lipolytic activity should be demonstrable even in the simplest media compatible with growth of the organisms; such ferment activity, however, would not necessarily rule out the latter possibility.

The experiments here recorded were designed to show the effect of the composition of the medium on the nature and extent of lipolytic activity exhibited by a rapidly growing human tubercle bacillus (597). The media selected for this purpose varied in composition from one of extreme simplicity, containing ammonium chlorid as a source of nitrogen, and ethyl alcohol as a source of carbon, through others of various

1. See preceding articles.

degrees of complexity to the regulation nutrient broth commonly used for the culture of bacteria, nutrient meat-juice-peptone broth. The qualitative composition of these media is clearly indicated in Table 1, where the sources of nitrogen and carbon are specifically set forth. The composition of the nutrient broths, except for the addition of various carbohydrates, is omitted for obvious reasons. The organism was grown for several successive transfers in each of these media before the lipolytic activity was tested, both to acclimatize it to the various ingredients and to insure maximum growth. The final determinations recorded below were made uniformly after twenty-one days' incubation at 37 C. The technic has been described previously.

Throughout this work controls have been made in the following manner:

Media Controls.—One cubic centimeter of the sterile uninoculated medium was mixed with 10 c.c. of sterile distilled water, and, after the introduction of the ester and toluene, incubated under parallel conditions with the corresponding lipase determinations in the same medium.

Ester Controls.—The appropriate amounts of ester and toluene suspended in 10 c.c. of water were likewise inoculated at 37 C. with the media controls and the lipase solutions. In order to save unnecessary complications in the table, it can be stated definitely that, without exception, both the media controls and the ester controls showed no change in reaction after incubating twenty-four hours in the manner indicated. That is to say, whatever changes are noticed in the lipase-containing solutions are due to the action of the ferment and not to any reactionary changes attributable either to the culture medium itself or in the esters.

Vigorous attention to the cleanliness of all apparatus coming in contact with the culture media has been observed. The organism was grown for several successive transfers in the medium in which the observations were subsequently made. This is a point of considerable importance, for the procedure eliminates the possibility of transfer of active substance at the time of inoculation.

With this explanation, the results shown in Table 1 are self-explanatory. The figures in the table represent the cubic centimeters of N/50 NaOH required to neutralize the acid liberated from the various esters after twenty-four hours' incubation with 1 c.c. of the clear medium underlying the pellicle of bacteria, which forms as the organisms grow. The total lipase activity of each culture is, therefore, theoretically, one hundred times the amount indicated in the table, for it will be remembered that but 1 c.c. of the culture medium is used in these determinations, while the total volume of culture in which the organisms have grown is 100 c.c. in each case. Generally speaking, the results show that the extent of the lipolytic activity increases with

TABLE 1

Lipolytic Activity of Tubercle Bacillus 597

Days of Growth	Composition of Media			Cubic Centimeter of N/50 NaOH to Neutralize							
	Source of Nitrogen	Source of Carbon	Other Ingredients	Methyl Salicylate	Ethyl Acetate	Ethyl Butyrate	Amyl Acetate	Amyl Butyrate	Amyl Valerianate	Triacetin	Castor Oil
21	NH_4Cl	CH_3CH_2OH	Na_2HPO_4NaCl	....	....	0.25	....	....	....	....	....
21	NH_4Cl	Glycerin	Na_2HPO_4NaCl	....	0.20	0.40	0.70	0.40	0.50	....	....
21	NH_4Cl	Dextrose	Na_2HPO_4NaCl	....	0.20	0.35	0.40	0.65	0.45	....	....
21	NH_4Cl	Mannite	Na_2HPO_4NaCl	....	0.30	0.40	0.40	0.65	0.55	....	....
21	Glucose	Amine	Na_2HPO_4NaCl	....	....	0.25	....	....	....	....	....
21	$(NH_4)_2HPO_4$	CH_3CH_2OH	NaCl	....	....	0.25	....	....	....	....	....
21	$(NH_4)_2HPO_4$	CH_3COONa	NaCl	....	0.55	0.55	0.60	0.45	0.50	....	....
21	$(NH_4)_2HPO_4$	Glycerin	NaCl	0.20	0.40	0.30	0.40	0.20	0.35	....	0.35
21	$(NH_4)_2HPO_4$	Dextrose	NaCl	0.20	0.25	0.40	0.45	0.20	0.40	....	0.40
21	$(NH_4)_2HPO_4$	Mannite	NaCl	0.25	0.30	0.55	0.25	0.50	0.50	....	0.50
21	Asparagin	CH_3CH_2OH	Na_2HPO_4NaCl	....	....	0.30	....	....	....	....	....
21	Asparagin	Glycerin	Na_2HPO_4NaCl	0.40	0.85	0.60	0.70	0.40	0.30	0.40	0.90
21	Asparagin	Dextrose	Na_2HPO_4NaCl	0.50	0.70	0.70	0.80	0.45	0.40	0.50	0.40
21	Asparagin	Mannite	Na_2HPO_4NaCl	0.50	0.70	1.10	0.95	0.50	0.25	0.50	0.50
21	Asparagin $(NH_4)_2HPO_4$	CH_3CH_2OH	Na_2HPO_4NaCl	....	....	0.30	....	....	....	....	....
21	Asparagin $(NH_4)_2HPO_4$	Glycerin	Na_2HPO_4NaCl	0.60	0.80	0.55	0.80	0.50	0.70	....	....
21	Asparagin $(NH_4)_2HPO_4$	Dextrose	Na_2HPO_4NaCl	0.30	0.65	0.45	1.00	0.65	0.85	....	....
21	Asparagin $(NH_4)_2HPO_4$	Mannite	Na_2HPO_4NaCl	0.50	0.80	0.60	0.85	0.45	0.75	....	....
21	Nutrient broth	Plain	....	....	....	0.85	....	....	....	....	....
21	Nutrient broth	Glycerin	....	....	....	1.95	....	....	....	....	....
21	Nutrient broth	Dextrose	....	....	....	1.55	....	....	....	....	....
21	Nutrient broth	Mannite	....	....	....	1.75	....	....	....	....	....
..	Control	....	....	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

increased complexity in composition of the medium in which the organism is grown. The lipolytic activity, observed in media in which ammonia, either as ammonium chlorid or diammonium hydrogen phosphate, is a source of nitrogen, is distinctly less than that observed in nutrient broth where amino-acids, and probably other nitrogen-containing bodies, are possible sources of nitrogen. The source of carbon appears to make but little difference in the extent of lipolytic activity, at least in the simpler media where this relationship can be definitely observed. The relative luxuriance of growth of the tubercle bacillus in the various media presented above leads one to believe that the extent and thickness of the pellicle, that is, the luxuriance of the growth of the organism, varies almost directly with the increasing complexity in

TABLE 2
EFFECT OF HEATING LIPASE TO 100 C. FOR 15 MINUTES

Days of Growth	Source of Nitrogen	Source of Carbon	Other Ingredients	Unheated Filtrate Cubic Centimeters of N/50 NaOH					Heated Filtrate (100 C.-15') Cubic Centimeter of N/50 NaOH				
				Ethyl Acetate	Ethyl Butyrate	Amyl Acetate	Amyl Butyrate	Amyl Valerianate	Ethyl Acetate	Ethyl Butyrate	Amyl Acetate	Amyl Butyrate	Amyl Valerianate
22	$(NH_4)_2HPO_4$	Sod. Lactate	NaCl	0.55	0.60	0.60	0.45	0.50	0.60	0.50	0.35	0.45	0.50
22	$(NH_4)_2HPO_4$	Glycerin	NaCl	0.40	0.50	0.70	0.40	0.50	0.45	0.40	0.80	0.30	0.60
22	$(NH_4)_2HPO_4$	Dextrose	NaCl	0.40	0.35	0.40	0.65	0.45	0.25	0.50	0.70	0.35	0.45
22	$(NH_4)_2HPO_4$	Mannite	NaCl	0.30	0.35	0.40	0.65	0.55	0.25	0.45	0.50	0.45	0.60

composition of the medium. Therefore, the conclusion that the extent of lipolytic activity varies almost directly with the relative luxuriance of growth appears to be justified. On the other hand, the action of the lipase is about the same on the various esters tested, irrespective of the composition of the medium. This observation is in harmony with the extensive observations of Loevenhart,[2] who has shown that the same lipase can act on a variety of esters.

It is apparent from the results that the lipase produced by the tubercle bacillus can act on various esters, even tho it is developed by the organisms grown in media of the simplest composition. It should be again emphasized that the organisms were repeatedly trans-

2. Jour. Biol. Chem., 1907, 2, p. 456.

ferred in these simple media before the results presented here were obtained, so that there is practically no possibility that a transfer of lipase, other than that developed by the organisms in the medium under question, can have taken place at the time of inoculation.

Table 2 shows the effect of heating the lipase solution to 100 C. for fifteen minutes before adding the ester and toluene. The results indicate that the lipase demonstrable in the simplest media is thermostabile, agreeing in this respect with the thermostability of the lipase found in the more complex nutrient broth. This is another argument in favor of the identity of the lipase developed in these various media.

CONCLUSIONS

A rapidly growing strain of the human tubercle bacillus produces a lipase which appears to be qualitatively the same when it is grown in media varying in composition from one consisting essentially of ammonium chlorid, ethyl alcohol, Na_2HPO_4, and NaCl, to the extremely complex nutrient meat-juice-peptone broth ordinarily used for bacterial cultures.

The lipase observed in the simplest media acts on various esters, irrespective of the nature of the carbon compound of the medium in which it is developed. For example, the lipase developed in the $(NH_4)_2HPO_4$ mannite medium acts on triacetin and castor oil as energetically as on ethyl butyrate, or other simple esters. That is to say, the lipase developed in the simplest medium acts even on a complex glycerid.

This lipase appears to be thermostabile whether it is tested in the simplest media or in the most complex.

The activity of the lipase appears to be roughly proportionate to the relative luxuriance of the growth of the tubercle bacillus.

A COMPARISON OF THE CURVES OF LIPOLYTIC ACTIVITY AND PROTEOLYSIS OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN MEDIA OF VARIED COMPOSITION

STUDIES IN ACID-FAST BACTERIA. IX

A. I. Kendall, A. W. Walker, and A. A. Day

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

In the previous articles it has been shown that broth cultures of various acid-fast bacteria exhibit lipolytic activity; that this lipolytic activity is also demonstrable in the autolyzed bacteria from the same media; and that the lipase in solution in the broth and in the bodies of the bacteria grown in this broth is thermostabile. Furthermore, this lipase, irrespective of the composition of the medium in which it is produced, acts on various esters and glycerids. The extent of lipase activity observed, both in culture media and in autolyzed bacteria taken from these media, appears to vary with the luxuriance of the growth of the organisms. This observation, however, appears to apply more strictly to the filtered broth cultures than to the autolyzed bacteria obtained from these cultures, and the question arises: Is this lipolytic activity proportionate to the amount of autolysis which the bacteria undergo in the media, or does this lipolytic activity vary with the vegetative activity (metabolism) of the organisms? The solution of this question possesses more than academic interest, for it is conceivable that an active exolipase might play a not unimportant part in the development of the tubercle bacillus in the human body.

If the former possibility alone were realized, tubercle bacilli should exhibit lipolytic activity more or less proportionate to their autolysis; whereas, if the latter possibility alone (or associated with the former possibility) were involved, there would be a rough parallelism between the amount of lipolysis demonstrable in culture and the extent of vegetative activity of the organisms themselves in the same culture, provided appropriate media were used. That is to say, if the tubercle bacillus produces an exolipase, it is probable that the curves of lipolytic activity and vegetative activity would reach their maxima more or less synchronously.

TABLE 1

METABOLISM OF TUBERCLE BACILLI IN MEDIUMS A, B, AND C

		Dextrose					Mannite					Glycerin				
Medium	Days	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH
A	14	—0.10	—4.2	—10.0	0.25	......	—0.20	—4.2	—10.0	0.30	......	0.00	—4.2	—10.0	0.40	....
	21	—0.15	—4.2	—10.0	0.40	0.40	—0.40	—4.2	—10.0	0.55	0.40	+0.10	—4.9	11.66	0.30	0.45
	28	—0.05	—2.8	6.56	0.35	0.25	—0.20	—2.1	—5.0	0.35	0.35	+0.30	—4.2	10.0	0.25	0.30
B	14	—0.05	+4.9	9.0	0.20	......	—0.20	+16.1	29.5	0.25	....	0.00	+4.2	15.4	0.30	....
	21	—0.05	+13.3	24.4	0.50	0.10	—0.20	+20.3	37.2	0.30	0.05	+0.10	+7.0	12.8	0.40	0.00
	28	—0.05	+23.8	43.5	0.75	0.15	0.00	+21.0	38.4	0.65	0.20	+0.10	+12.6	23.1	0.60	0.35
C	14	—0.20	+9.1	10.2	0.45	......	0.00	+23.1	25.8	0.60	....	—0.50	+4.2	4.62	0.55	....
	21	—0.20	+18.9	21.1	0.70	0.75	0.00	+23.1	25.8	1.00	0.60	—0.30	+14.0	15.4	0.50	0.50
	28	0.00	+16.1	18.0	0.45	0.20	—0.20	+26.6	29.7	0.90	0.45	+0.10	+17.5	19.3	0.85	0.55

* Figures expressed as NH_3 per 100 c.c. (Medium A) represent the total soluble nitrogen of the clear fluid underlying the bacterial growth: full details in text.

TABLE 2

GROWTH OF TUBERCLE BACILLI IN MEDIUM D

		Dextrose					Mannite					Glycerin				
Bacillus Tuber-culosis	Days	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH
WI	21	+0.40	23.8	32.1	0.50	....	+0.20	30.8	46.8	0.60	....	+0.30	9.8	15.2	0.60	....
	28	+0.30	36.4	49.1	0.70	0.65	+0.20	39.9	60.3	0.95	0.65	+0.30	28.0	43.4	0.60	0.55
	35	+0.20	30.8	41.5	0.45	0.15	+0.30	30.8	46.8	0.70	0.15	0.00	30.8	47.8	0.45	0.25
	42	+0.10	34.3	46.3	0.50	0.15	+0.10	31.5	48.0	0.75	0.10	+0.10	33.6	52.1	0.40	0.10
WII	21	0.00	14.0	18.9	0.50	....	+0.20	31.5	48.0	0.70	....	+0.20	14.0	21.8	0.60	....
	28	+0.20	36.0	49.1	0.65	0.65	+0.10	37.8	57.5	0.95	0.55	+0.20	27.3	42.4	0.55	0.80
	35	+0.30	31.5	42.4	0.60	0.30	0.00	30.8	46.8	0.70	0.10	+0.10	32.2	50.0	0.45	0.40
	42	+0.30	32.6	45.3	0.50	0.10	+0.10	33.6	51.1	0.60	0.10	+0.10	35.0	54.4	0.40	0.10
597I	21	+0.50	18.2	24.5	0.50	....	+0.10	30.1	45.8	0.60	....	+0.20	23.8	37.6	0.50	....
	28	+0.50	32.9	44.3	0.70	0.40	+0.10	39.9	60.3	1.10	0.50	+0.20	25.2	39.3	0.60	0.90
	35	+0.50	30.1	40.5	0.45	0.25	+0.10	32.2	49.0	1.45	0.20	+0.20	28.0	43.4	0.40	0.20
	42	+0.20	32.2	43.4	0.40	0.25	0.00	31.5	48.0	0.55	0.20	+0.40	31.5	49.7	0.35	0.15
597II	21	+0.30	17.5	23.6	0.50	....	+0.10	30.1	45.8	0.70	....	+0.30	10.5	16.3	0.55	....
	28	+0.30	31.5	42.4	0.75	0.55	+0.10	39.9	60.3	1.05	0.65	+0.30	25.2	39.3	0.40	0.50
	35	+0.30	31.5	42.4	0.45	0.20	+0.10	31.5	48.0	0.25	0.20	+0.10	23.8	37.6	0.25	0.25
	42	+0.30	31.5	42.4	0.60	0.15	+0.20	31.5	48.0	0.65	0.15	+0.30	32.2	50.0	0.30	0.20

With these two possibilities in view, the following experiments were undertaken to demonstrate the relationship, if such exists, between the lipolytic activity of cultures of certain acid-fast organisms and the metabolism of these organisms, as measured by ammonia formation and a change in reaction. In the present communication the metabolism of two rapidly growing human tubercle bacilli is considered. The procedures followed are precisely those described in previous communications where the full details are given.

Briefly, the metabolism of the tubercle bacilli (vegetative activity) was measured by the changes in ammonia content of the medium, which indicates the action of the organisms on the protein constituents, and the changes in reaction to phenolphthalein. The lipase activity was measured according to the method described previously. It consisted, essentially, in suspending 1 c.c. of the bacteria-free filtrate of the various cultures in freshly boiled, distilled water, adding 0.25 c.c. of ethylbutyrate and 0.5 c.c. of toluene, and incubating at 37 C. together with appropriate controls. The increase in acidity in terms of N/50 NaOH is taken as a measure of the lipolytic activity of the culture for the period of incubation mentioned.

The organisms have been studied in media of extremely simple composition, and through successive degrees of complexity to ordinary nutrient broths with various sources of carbon, as follows:

Medium A (Table 1).—$(NH_4)_2$ HPO_4, 4 gm., and NaCl, 5 gm., in 1,000 c.c. redistilled water with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

It will be observed that the determination "Ammonia" (Table 1) is in reality the determination of the total soluble nitrogen in this medium, for all the nitrogen of $(NH_4)_2HPO_4$ is measured by the Folin Air Current Method of ammonia estimation. The decrease in "ammonia," therefore, observed during the second and third weeks of incubation, represents the amount of nitrogen appropriated by the bacteria as they increased in numbers. The reappearance of nitrogen, observed at the end of the fourth week in the filtrates of the cultures in this medium (associated with a decrease in lipolytic activity of the solution), is probably to be regarded as an indication of autolysis of the bacteria with the liberation from them of nitrogenous substances which again pass into solution, and are recovered as "ammonia."

Medium B (Table 1).—Asparagin, 4 gm., Na_2HPO_4, 2 gm., and NaCl, 5 gm., in redistilled water, 1,000 c.c., with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

Medium C (Table 1).—Asparagin, 2 gm., $(NH_4)_2HPO_4$, 2 gm., Na_2HPO_4, 1 gm., NaCl, 5 gm. in 1,000 c.c. of redistilled water.

Medium D (Table 2).—Asparagin, 4 gm., $(NH_4)_2HPO_4$, 2 gm., NaCl, 5 gm. in 1,000 c.c. redistilled water.

Medium E (Table 3).—Fairchild's peptone (extracted with ether, alcohol, acetone, and petroleum ether to remove lipoids and fat in Medium E[a] and unextracted in Medium E[b]), 5 gm., Na_2HPO_4, 2 gm., NaCl, 5 gm. in 1,000 c.c. redistilled water, with 1 percent dextrose, or 3 percent glycerin.

Medium F (Table 4).—Regulation sugar-free meat-juice-peptone broth with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

As shown in Table 1, in the ammonium phosphate medium (A), the maximum nitrogen metabolism was reached on the twenty-first day, at which time the organisms had removed 10 percent of the total medium from solution; this nitrogen was probably incorporated in their bodies. Lipase activity is also maximum at this time. By the end of the twenty-eighth day, autolysis of the bacteria was well under way (shown by the reappearance of some of the nitrogen in solution) and lipolytic activity had diminished somewhat.

TABLE 3
Growth of Tubercle Bacilli in Medium E

Bacillus Tuberculosis	Days	Plain Broth (a)				Plain Broth (b)			
		Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH	Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH
597	3	—0.50	1.4	1.45	0.15	—1.30	11.9	5.30	0.20
	7	—1.30	23.8	17.9	0.10	—1.70	49.7	22.2	0.05
	14	—1.20	37.8	28.4	0.10	—1.80	64.4	28.7	0.40
	21	—1.30	35.0	26.3	0.15	—1.90	60.2	26.9	0.15
	28	—1.30	28.7	21.6	0.30	—1.90	52.5	23.4	0.45
W	3	—0.90	4.2	3.15	0.10	—1.40	11.9	5.32	0.20
	7	—1.30	29.4	22.1	0.10	—1.90	52.5	23.5	0.05
	14	—1.10	38.5	29.0	0.15	—1.70	65.1	29.0	0.45
	21	—1.40	35.0	26.3	0.10	—2.00	60.9	27.2	0.45
	28	—1.10	28.7	21.6	0.35	—1.70	43.8	21.5	0.50

In the asparagin medium (B) there is steady increase in metabolism in the dextrose, mannite, and glycerin modifications, respectively, associated with a progressive increase in lipase activity. This experiment was not carried far enough to show the recession in metabolism, but other studies with the same media indicate that at the end of the fourth week there is usually a recession of metabolism.

In the ammonium phosphate-asparagin medium (C) the maximum of metabolism and lipolytic activities is reached on the twenty-first day, except in the glycerin modification.

The general parallelism between metabolism and lipolytic activity (Medium D) is clearly set forth in Table 2.

Medium E (a) consists of Fairchild's peptone extracted to remove all fats and lipoids. Growth was fairly luxuriant, but lipase activity is

surprisingly low (Table 3). No explanation for this disparity can be advanced; certain other peculiarities—the production of a progressively alkaline reaction in glycerin and unusual products of growth—suggest that this peptone medium is acted on differently by the tubercle bacillus than the regulation meat-juice peptone media.

Medium F consists of regulation meat-juice-peptone broth, plain and with dextrose, mannite, or glycerin. The observations (Table 4) have been continued for fifty-one days; the maximal proteolytic activity is reached by the third week, at which time lipolytic activity appears to be at its height. There is a great diminution in the ammonia content

TABLE 3—(*Continued*)
Growth of Tubercle Bacilli in Medium E

Bacillus Tuberculosis	Days	Dextrose Broth (a)				Glycerin Broth (b)			
		Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{Total\ N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH	Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{Total\ N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH
597	3	—1.20	5.6	4.00	0.15	—0.50	3.5	2.63	0.10
	7	—1.40	1.40	1.00	0.10	—0.80	1.4	1.05	0.15
	14	—1.40	17.5	12.50	0.10	—0.80	—2.8	—2.1	0.15
	21	—1.50	23.1	16.50	0.15	—0.80	—2.8	—2.1	0.20
	28	—1.40	23.8	17.0	0.50	—0.90	—2.8	—2.1	0.10
W	3	—1.30	6.3	4.50	0.15	—0.60	2.1	1.58	0.15
	7	—1.20	6.3	4.50	0.30	—0.60	3.5	2.63	0.10
	14	—1.50	29.4	21.0	0.95	—0.80	—0.70	—0.52	0.15
	21	—1.50	23.1	16.5	0.95	—0.70	—2.1	—1.58	0.15
	28	—1.40	23.8	17.0	0.90	—0.90	—2.8	—2.1	0.10

of the media after this time; at the end of fifty-one days' incubation there is actually less than at the start. The lipase activity declines considerably after three weeks, but not proportionately to the ammonia.

The recession of ammonia appears to be associated with autolysis of the bacteria, and in this connection the experiments of Lockemann[1] and Möllers[2] are suggestive. Lockemann showed that the weight of tubercle bacilli grown in glycerin broth increased steadily to a maximum, then diminished somewhat; and Möllers' observations would indicate that the antigenic content of the same culture paralleled the weight curve of Lockemann. It is a striking coincidence to compare these curves with the similar curves of metabolic and lipolytic activity,

1. Veröffentlichungen der Robert Koch, Stiftung zur Bekämpfung der Tuberculose, 1914, 10, p. 21.
2. Ibid., p. 56.

and the possibility suggests itself that these phenomena are closely related, if not identical, in origin.

The lipase curve, following the curve of proteolysis to its maximum, but diminishing far less rapidly during the period of recession, speaks strongly in favor of the view advanced in a previous article, that the lipase is an exoferment, in part at least. Otherwise lipase activity should increase progressively with autolysis, unless its activity is inhibited by the accumulation of products of its own production.

TABLE 4

Growth of Tubercle Bacillus W in Medium F

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.10	0.00	0.00	0.15	—0.50	—0.70	—0.22	0.15
3	—0.30	0.70	0.22	0.20	—1.10	—2.80	0.88	0.55
6	—0.90	2.8	0.88	1.90	—1.60	1.40	0.44	0.75
10	—1.40	16.8	5.22	1.60	—1.60	14.70	4.57	2.50
15	—1.70	22.4	6.96	1.65	—2.10	28.7	8.60	1.75
21	—1.70	26.6	8.30	1.90	—1.80	34.3	10.65	2.60
28	—1.10	14.0	4.40	1.40	—1.70	26.6	8.30	1.40
36	—2.10	5.6	1.74	1.25	—2.40	9.8	3.04	1.05
43	—1.60	0.00	0.00	1.20	—2.50	7.0	2.17	1.10
51	—1.50	—2.8	—0.87	1.15	—2.10	—5.6	—1.74	1.00

It was a point of some interest to determine just how much nitrogen is contained in the bacterial cells at the end of fifty-one days' incubation. This was readily accomplished by comparing the total soluble nitrogen in media after fifty-one days' growth with the initial total nitrogen of media of the same composition inoculated under parallel conditions. The tabulated results show clearly that between 21 and 32 percent of the initial nitrogen is not in solution, and the logical inference is that this "lost" nitrogen is retained in the bodies of the bacteria.

Bacillus Tuberculosis W	Plain	Dextrose	Mannite	Glycerin
Initial total N_2—mg.	322	322	322	322
Final total N_2—mg.	231	217	224	252
Loss total N_2—mg.	91	105	98	70
Percent loss*	28.3	32.6	30.4	21.7

*The percentage of nitrogen not in solution, but incorporated in the bodies of the bacteria which had grown in 51 days.

There is a noteworthy parallelism between the curve of vegetative activity, as measured by ammonia formation, and the curve of lipolytic activity, as measured by the changes in acidity in all media. The period of greatest ammonia production, which is assumed to mark the period of maximum vegetative activity, coincides definitely with the period of greatest lipolytic activity; and the recession of ammonia, which has been commented on in the previous communications, appears to be associated with a decrease in the lipolytic activity of the cultures.

TABLE 4—(*Continued*)
Growth of Tubercle Bacillus W in Medium F

	Mannite Broth				Glycerin Broth			
Days	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.20	0.00	0.00	0.00	0.00	—1.4	—0.44	0.05
3	—0.40	0.70	0.22	0.40	—0.30	1.4	0.44	0.30
6	—0.80	6.3	1.95	1.85	—0.70	4.9	1.21	2.05
10	—0.70	16.8	5.22	2.20	—0.60	8.4	2.61	2.45
15	—0.80	28.7	8.90	1.80	—0.70	18.2	5.65	2.30
21	—0.70	29.4	9.12	1.65	—0.30	18.9	5.87	3.80
28	—0.60	25.9	8.05	1.45	+0.10	18.9	5.87	1.35
36	—0.60	25.2	7.83	1.30	—0.30	16.8	5.22	1.15
43	—0.50	21.7	6.74	1.35	0.00	11.2	3.48	1.25
51	—0.50	8.4	2.60	1.40	—0.20	—0.70	—0.21	1.15

CONCLUSIONS

The period of maximum vegetative activity of broth cultures of certain avirulent, rapidly growing tubercle bacilli, as measured by ammonia formation (proteolysis), appears to coincide with the period of maximum lipolytic content of these cultures, as measured by their action on ethylbutyrate.

Both ammonia production and lipolytic activity are extremely slight during the first day's growth of these organisms, and increase, roughly, proportionately to their respective maxima.

There is a noteworthy recession of both factors after this maximum is reached.

These experiments appear to warrant the assumption that the organisms studied excrete a soluble, active lipase during the period of active development; for if autolysis alone were responsible for the lipolytic activity observed in these cultures, it should increase as autolysis proceeds.

A COMPARISON OF THE CURVES OF LIPOLYTIC ACTIVITY AND PROTEOLYSIS OF CERTAIN ACID-FAST BACILLI IN NUTRIENT BROTHS

STUDIES IN ACID-FAST BACTERIA. X

A. I. KENDALL,
A. W. WALKER,
AND
A. A. DAY

Reprinted from
THE JOURNAL OF INFECTIOUS DISEASES, Vol. 15, No. 3, Nov. 1914, pp. 467-471

A COMPARISON OF THE CURVES OF LIPOLYTIC ACTIVITY AND PROTEOLYSIS OF CERTAIN ACID-FAST BACILLI IN NUTRIENT BROTHS

STUDIES IN ACID-FAST BACTERIA. X

A. I. Kendall, A. W. Walker, and A. A. Day

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

The demonstration of an active lipase in solution in broth cultures of certain rapidly growing human tubercle bacilli, the curve of lipolytic activity of which, measured in terms of liberation of acid from ethylbutyrate, follows rather closely their curve of protein metabolism,[1] suggests that this lipase may play a part in the preparation of certain nutritional substances for assimilation by these bacteria. There is a certain amount of presumptive evidence in favor of this view, for the lipase appears to be active even after the organisms are removed from the media in which it is developed, and it is most abundant or most active when bacterial development is intense. Furthermore, this lipase acts on a variety of esters and glycerids,[2] which fact, altho not proving its exogenous functions, suggests that it is sufficiently versatile in its attack to split those substances of a fatty nature it might be confronted with. If this lipase is indeed a true exoferment, it might be justifiable to assume that other bacteria of the same type as the tubercle bacillus (other acid bacteria) would also elaborate such a ferment.

The observations recorded here indicate that filtrates of broth cultures of certain acid-fast bacilli, other than the human tubercle bacillus, do exhibit such lipolytic activity.

The organisms studied in this connection were the so-called leprosy bacillus (Duval), the grass bacillus, and the smegma bacillus. The first organism is of unknown history; the last two were received from Professor Winslow of the American Museum of Natural History. The technical details of measuring the lipolytic activity of such cultures has been described in detail previously,[3] and will not be repeated here.

1. See preceding article.
2. Study VIII.
3. Study VI.

TABLE 1
LIPOLYTIC ACTIVITY OF SMEGMA BACILLUS

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.50	9.1	2.83	0.00	—0.90	8.4	2.61	0.10
3	—1.10	0.7	0.22	0.15	—1.10	7.0	2.2	0.60
6	....	....	....	....	—1.90	5.6	1.74	1.15
10	—1.50	11.2	3.48	0.60	—1.10	18.9	5.87	1.75
15	—1.50	19.6	6.08	1.30	—1.10	22.4	6.96	1.75
21	—1.60	7.7	2.39	0.80	—2.10	28.0	8.70	1.95
28	—1.40	7.7	2.39	0.50	—2.10	20.3	6.30	1.00
36	—1.30	0.00	0.00	0.50	—1.20	23.8	7.40	1.10
43	—1.80	4.2	1.30	0.60	—2.40	18.2	5.66	1.60
52	....	....	....	....	—2.20	—7.7	—2.39	1.30

TABLE 2
LIPOLYTIC ACTIVITY OF GRASS BACILLUS III

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.10	0.7	0.22	0.05	—0.70	9.8	3.04	0.20
3	—0.20	1.4	0.44	0.20	—0.90	0.7	0.22	0.40
6	—1.10	4.2	1.30	0.55	—1.80	0.7	0.22	0.75
10	—1.70	14.7	4.57	0.60	—1.60	5.6	1.74	1.40
15	—1.50	24.5	7.60	1.60	—2.00	23.8	7.40	1.75
21	—2.00	28.3	7.40	1.80	—2.20	21.7	6.75	1.65
28	—1.60	20.3	6.30	0.90	—2.20	16.1	5.00	0.80
36	—1.90	7.0	2.20	1.00	—2.30	8.4	2.60	1.05
43	—1.80	1.4	0.44	1.10	—2.40	1.4	0.44	1.15
52	—2.00	—4.9	—1.52	1.30	—2.10	—4.9	—1.52	1.15

TABLE 3
LIPOLYTIC ACTIVITY OF DUVAL'S LEPROSY BACILLUS

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	0.00	0.00	0.00	0.05	—0.50	—0.7	—0.22	0.00
3	—0.20	0.00	0.00	0.05	—0.60	—1.4	—0.44	0.10
10	—1.20	0.00	0.00	0.05	—1.20	0.00	0.00	0.00
21	—0.70	0.70	0.22	0.20	—1.00	—1.4	—0.44	0.00
28	—1.50	—2.8	—0.87	....	—0.90	—12.6	—3.92	0.20
43	—2.20	—12.6	—3.92	0.20	—2.20	—16.8	—5.20	0.10
52	—1.90	—11.9	—3.70	0.10	—2.20	—14.7	—4.56	0.10

TABLE 1—(*Continued*)
Lipolytic Activity of Smegma Bacillus

	Mannite Broth				Glycerin Broth			
Days	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.30	8.4	2.60	—0.05	—0.30	—0.7	—0.22	0.05
3	—0.70	0.00	0.00	0.05	—0.20	—0.7	—0.22	0.05
6	—1.30	0.00	0.00	0.10	—0.50	4.2	1.30	0.95
10	—0.80	1.4	0 44	1.75	—0.50	—2.8	—0.87	1.10
15	—0.50	16.8	5.22	1.20	—0.30	2.8	0.87	1.10
21	—0.90	16.8	5.22	1.60	—0.60	12.6	3.91	1.55
28	—1.50	2.80	8.70	1.10	—0.60	14.0	4.40	1.80
36	—1.80	14.0	4.35	1.00	—0.60	0.7	0.22	1.05
43	—1.60	19.6	6.04	1.65	0.00	0.7	0.22	1.75
52	—2.00	—5.6	—1.74	1.30	—0.40	—4.9	—1.52	1.40

TABLE 2—(*Continued*)
Lipolytic Activity of Grass Bacillus III

	Mannite Broth				Glycerin Broth			
Days	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.40	9.8	3.04	0.10	—0.10	7.7	2.39	0.20
3	—0.40	0.00	0.00	0.15	—0.30	0.00	0.00	0.25
6	—0.70	0.7	0.22	0.75	—1.00	0.00	0.00	0.05
10	—0.90	1.4	0.44	1.60	—0.40	7.7	2.39	1.00
15	—1.10	8.4	2.60	1.80	—0.30	13.3	4.13	1.70
21	—1.30	15.4	4.78	1.80	—0.30	13.3	4.13	1.60
28	—1.30	11.9	3.70	1.30	—0.30	16.8	5.22	1.20
36	—1.40	16.1	5.00	1.40	0.10	15.4	4.78	1.10
43	—1.80	2.8	0.88	1.60	0.00	4.9	1.52	1.05
52	—1.80	—4.9	—1.52	1.30	—0.20	1.4	4.30	1.25

TABLE 3—(*Continued*)
Lipolytic Activity of Duval's Leprosy Bacillus

	Mannite Broth				Glycerin Broth			
Days	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.10	9.8	+3.04	0.00	0.00	1.4	0.44	0.10
3	—0.30	0.00	0.00	0.05	—0.10	1.4	0.44	0.10
10	—0.80	—4.9	—1.52	0.00	—0.50	—0.7	—0.22	0.05
21	—0.90	—4.9	—1.52	0.00	—0.80	—5.6	—1.74	0.05
28	—0.60	—9.8	—3.04	....	—1.30	—9.1	—2.82	....
43	—1.40	—6.3	—1.95	0.05	—1.30	—11.2	—3.48	0.10
52	....	....	....	....	—1.70	—13.3	—4.13	0.10

The tables indicate that, with the exception of the Duval organism, broth cultures of these acid-fast bacteria do exhibit lipolytic activity; furthermore, there is a tendency for the maximum period of proteolysis to coincide with the period of maximum lipolytic activity. The organisms, however, present certain peculiarities in their metabolism curves which deserve further consideration before their exact significance is set forth, and for this reason only the most general statements are warranted.

TABLE 4

The Initial Total Nitrogen and the Residual Total Nitrogen of Various Media after the Organisms Had Grown in Them for 52 Days

	Smegma Bacillus	Lepra Bacillus	Grass Bacillus
Plain broth—			
Initial total N_2—mg.	—	322	322
Final total N_2—mg.	—	266	231
Loss total N_2—mg.	—	56	91
*Percent loss	—	17.4	28.3
Dextrose broth—			
Initial total N_2—mg.	322	322	322
Final total N_2—mg.	224	266	231
Loss total N_2—mg.	98	56	91
*Percent loss	30.4	17.4	28.3
Mannite broth—			
Initial total N_2—mg.	322	—	322
Final total N_2—mg.	238	—	245
Loss total N_2—mg.	84	—	77
*Percent loss	26.1	—	23.9
Glycerin broth—			
Initial total N_2—mg.	322	322	322
Final total N_2—mg.	301	112	266
Loss total N_2—mg.	21	210	56
*Percent loss	6.51	6.52	17.4

* Percent loss = percentage of initial total nitrogen which has been appropriated into the bacteria as they have increased in the medium. This nitrogen fraction is subject to variations, increasing as growth increases, and decreasing as vegetative activity wanes and autolytic processes cause partial solution of the bacterial cells.

The Duval bacillus reacts quite differently from the others; altho it grew luxuriantly in all the media, the lipase activity demonstrable in culture appears to be practically nil. Further observations with this organism are in progress.

An approximate measure of the luxuriance of growth of these bacteria, at any stage of the history of the culture, may be obtained by determining the amount of nitrogen which has been tied up in their bodies incidental to their growth. This is readily accomplished by comparing the total nitrogen of the uninoculated medium with that of the clear medium underlying the firm, tenacious pellicle of bacteria which have grown in media of the same composition. Table 4 shows the initial total nitrogen and the residual total nitrogen in milligrams

per 100 c.c. of various media after the organisms had grown in them for fifty-two days. (These bacteria were grown in media of 100 c.c. volume.)

In general, with the exception of the "lepra bacillus," the acid-fast bacteria discussed show a parallelism in their curves of lipolytic activity, as measured by the liberation of acid from ethylbutyrate and their curves of proteolysis. This parallelism is discernible in cultures in plain, dextrose, mannite, and glycerin broths.